Brain, Gut, and Immune Interactions in Autism Spectrum Disorder

Thesis by

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Abstract

Autism spectrum disorder (ASD) is a class of complex neurodevelopmental disabilities that are characterized by the presence and severity of stereotyped behaviors, impaired communication, and abnormal social interactions. The incidence of autism has rapidly increased to 1 in 88 children in the United States, making ASD one of the most significant medical and social burdens of our time. However, drugs are often used to treat autism-*related* conditions, including anxiety, hyperactivity, epilepsy, and obsessive-compulsive behaviors, and therapies for treating the core symptoms of autism are limited. Moreover, molecular diagnostics are not available for the reproducible identification of ASD; as yet, the disorder is diagnosed based on standardized behavioral assessments. Much research into ASD has focused on genetic, behavioral, and neurological aspects of the illness. However, primary roles for environmental risk factors and peripheral disruptions, such as immune dysregulation and gastrointestinal distress, have gained significant attention.

The work described in this thesis uncovers molecular mechanisms involved in the pathogenesis of autism-related endophenotypes in a mouse model of a primary autism risk factor, maternal immune activation (MIA). MIA is founded upon the strong epidemiological link between maternal infection and increased autism risk in the offspring. This risk factor can be translated to a mouse model with face and construct validity for autism, wherein pregnant mice injected with the immunogenic, double-stranded RNA poly(I:C) yield offspring with the core behavioral and neuropathological features of autism. Specifically, we report that MIA critically alters placental immune

status and endocrine function, reflecting a key pathway by which fetal development may be disrupted to manifest in ASD-related phenotypes. We identify signature changes to the fetal brain transcriptome in response to multiple modes of MIA, highlighting a converging pathway involved in the development of autism-related behaviors and neuropathologies. We characterize peripheral, neural, and enteric immune alterations in MIA offspring and uncover an immune contribution to autism-related behavioral abnormalities. Finally we demonstrate that a microbe-based therapeutic can ameliorate intestinal pathology, metabolic function, and autism-related behaviors in MIA mice, which supports a role for the gut-immune-brain axis in ASD.

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Chapter 1

Thesis summary

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Autism spectrum disorder (ASD) is a devastating neurodevelopmental ailment that encompasses five distinct conditions: autism, Asperger's syndrome, Rett syndrome, childhood disintegrative disorder and pervasive developmental disorder not otherwise specified (PDD-NOS). The disorders are diagnosed based on the presence and severity of three core symptoms: impairments in reciprocal social interactions, abnormal development and use of language, and repetitive, stereotyped behaviors or insistence on sameness. However, ASD is co-morbid with several other neurological symptoms, including intellectual disability, epilepsy, anxiety and mood disorders, as well as other peripheral symptoms, such as hyperserotonemia, immune dysregulation, and gastrointestinal abnormalities. The prevalence of autism has risen to 1 in 88 children in the United States, with males being more susceptible than females. The causes of autism are largely unknown, but are believed to originate from a combination of genetic and environmental risk factors. Several rare, single gene mutations have been associated with autism, including those affecting genes like *SHANK3, CNTNAP2* and *MET*, and environmental risk factors, such as the use of the teratogens valproic acid and thalidomide during pregnancy and the focus of my doctoral research, maternal immune activation (MIA), have been shown to increase the risk for features of autism in the offspring.

Maternal infection is a principal non-genetic cause of autism. Several large epidemiological studies have linked maternal infection during pregnancy with elevated risk for autism in the offspring. After the 1964 Rubella pandemic, 8-13% of children born to infected mothers developed features of autism, representing an over 200-fold increase in incidence of autism at the time. In a recent seminal study that included all children

born in Denmark from 1980 to 2005, a very significant association was found between autism and maternal viral infection during the first trimester of pregnancy. A similar association was found in another large study that included all residents of Stockholm County, Sweden from 2001-2007, where an analysis of over 4000 autism cases found a significant association between autism and maternal hospitalization for infection. By modeling this primary autism risk factor in mice, we are able to recapitulate the core behavioral and neuropathological of features of autism with face and construct validity. Studying these mice, which display clinically relevant features of autism, offers the unique potential to uncover the molecular mechanisms involved the pathogenesis of autism endophenotypes, and to further use this information toward the development of novel treatments for core behaviors and symptoms relevant to human autism.

The doctoral research presented in the following chapters can be broadly classified under three primary questions in the field of autism research: i) what causes autism?, ii) how is autism related to its co-morbid conditions?, iii) how can autism be treated? In Chapters 6-8, we trace the early molecular responses that mediate the effects of MIA on fetal development. In Chapters 4, 8 and 9 we characterize the later-life consequences of MIA on brain and behavior. In Chapters 11 and 12, we highlight immune dysregulation and gastrointestinal dysfunction as conditions that are highly co-morbid with autism, and we address the question of whether these co-morbidities contribute to the pathogenesis of autism-related endophenotypes. Finally in Chapter 12 we present data demonstrating that a microbe-based treatment ameliorates autism-related gastrointestinal disruptions, metabolome changes and behavior in MIA offspring.

Chapter 2 introduces autism as a complex neurodevelopmental disability and

provides a detailed overview of genetic, environmental and lesion models for autism, in addition to background on how features of autism are modeled in rodents. This content was published in 2011 in Autism Spectrum Disorders (Oxford University Press, Eds. Amaral DG, Dawson G, Geschwind DH). Chapter 3 discusses maternal infection as a risk factor for autism and schizophrenia, along with the several different animal models of maternal infection that are used to recapitulate features of autism and schizophrenia in rodents. This text was published in 2010 in Maternal Influences on Fetal Neurodevelopment: Clinical and Research Aspects (Humana Press, Eds. Zimmerman AW, Connors SJ).

Chapter 4 introduces the animal model of maternal infection that is used as a basis for the majority of my doctoral research. We show that offspring of poly(I:C)-injected mothers exhibit the three core diagnostic features of human autism: deficient communication, decreased social interaction and increased stereotyped behavior. This validates this poly(I:C) paradigm as a mouse model with face and construct validity for autism. The content was published in *Brain, Behavior and Immunity* in 2012.

Chapter 5 highlights the placenta as a key regulator of maternal-fetal interactions during pregnancy and reviews literature supporting a role for placental dysfunction in altering neurodevelopment. This serves as a preface to Chapter 6, in which we demonstrate that the cytokine IL-6, found to be a critical mediator of the effects of MIA on the development of autism-related behaviors in the offspring, regulates placental immune status and endocrine function. Interestingly, IL-6 action is necessary to drive the direct transfer of the MIA response to fetally-derived cells in the placenta. The review was published in *Developmental Neurobiology* in 2012 and the primary research article

was published in *Brain, Behavior and Immunity* in 2011.

Chapter 7 addresses the role of MIA on gene expression in the fetal brain and uncovers a converging molecular pathway common among three different modes of MIA. This transcriptome signature involves activation of crystalline family genes, which may trigger further molecular responses leading to impaired neurodevelopment in MIA offspring. This work was published in 2012 in *Translational Psychiatry*. Chapter 8 provides further evidence of early life alterations in brain development in MIA offspring, demonstrating that MIA neonates experience global changes in brain cytokines. Cytokine alterations are also observed at different time points and different brain regions in MIA versus control offspring. These results were published in 2012 in *Brain, Behavior and Immunity*. Chapter 9 reflects earlier work demonstrating alterations in hippocampal synaptic strength and plasticity in MIA versus controls, which correspond to alterations in nonspatial information processing. This work appeared in *Brain, Behavior and Immunity* in 2010.

In Chapter 10, we discuss increasing evidence supporting a role for immune dysregulation in ASD. We review the striking abnormalities observed in the neural, peripheral and enteric immune systems of autistic individuals, and explore how these changes could impact brain function. This serves as a preface to Chapter 11, wherein we report that MIA offspring, in addition to displaying the core behavioral and neuropathological features of autism, also exhibit autism-related immune abnormalities that suggest a pro-inflammatory-like state. Importantly, we explore whether these immune abnormalities contribute toward the development or persistence of autism-related behaviors in MIA offspring, by utilizing bone marrow transplants to reconstitute

immunity in immunologically-abnormal mice. Importantly, we provide evidence supporting the notion that immune changes in autism can contribute to primary behavioral features of the disorder. This work was published in *PNAS* in 2012, and the review in Chapter 10 is now in press in *Autism Open Access*.

In Chapter 12, we explore the use of a probiotic treatment, known to impact immune parameters in other disease models, to correct immune abnormalities in MIA offspring. Remarkably, we find that treatment with the human commensal *Bacteroides fragilis* corrects autism-related behaviors in MIA offspring. Surprisingly, however, this beneficial effect is not mediated by changes in immune status. This led us to uncover an autism-related gastrointestinal phenotype in MIA offspring, characterized by increased intestinal permeability and altered gut cytokine levels, in addition to a metabolic phenotype in MIA offspring involving global serum metabolome changes. Overall, we find that microbial treatment alters the composition of the gut microbiome in MIA offspring, which is associated with improvements in gastrointestinal physiology, changes in the systemic metabolome and amelioration of autism-related behaviors. This provides compelling evidence for a probiotic-based treatment for symptoms relevant to human autism. This work has been submitted for publication.

Finally, the appendices include preliminary research findings that are relevant to much of the research discussed in the primary chapters. Appendix A reflects experiments that explore the prenatal programming of placental hematopoietic stem cells as a basis for the persistent immune abnormalities observed in MIA offspring, as described in Chapter 11. Appendix B demonstrates that MIA leads to induction of the cytokine IL-6 in the fetal brain, and that basal levels of many brain cytokines depend on maternal IL-6 signaling.

Appendix C provides additional immune characterization of adult MIA offspring that was not included in the publication from Chapter 11. Appendix D assesses intestinal permeability and behavioral changes in a mouse model of early postnatal colitis, demonstrating that increased intestinal permeability itself is not sufficient to drive the autism-related changes in behavior reported in Chapter 12. In Appendix E, we provide preliminary data supporting a role for the commensal microbiota itself to contribute to communicative and sensorimotor behavior in wild-type mice. Lastly, in Appendix F, we explore a potential gene x environment interaction between two autism-related susceptibility factors: MHCI polymorphisms and maternal infection.

Chapter 2

Modeling features of autism in rodents

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Abstract

A variety of features of autism have been reproduced in rodents, including several hallmarks of abnormal behavior such as neophobia, deficits in social interactions, communication, stereotyped and repetitive motor behaviors, enhanced anxiety, abnormal pain sensitivity and eye blink conditioning, disturbed sleep patterns, seizures, and deficits in sensorimotor gating. There is also neuropathology that is frequently seen in autism such as a spatially restricted Purkinje cell deficit, as well as characteristic neurochemical changes (serotonin) and alterations in the immune status in the brain and periphery. Several known environmental risk factors for autism have been successfully established in rodents, including maternal infection and valproate administration, and genetic models are being produced that attempt to mimic some of the genetic variants associated with autism. There are also a few lesion models. This chapter critically reviews these various types of models, highlighting those with face and/or construct validity, and noting the potential for investigation of pathogenesis and therapies.

Points of interest

• A number of abnormal behaviors found in autism can be produced in rodent models. None of these behaviors are specific for autism, however.

• Human disorders caused by single-gene mutations that exhibit features of autism provide proof-of-principle for the role of genetics in autism. Similarly, studies showing that maternal thalidomide, valproate, or infection can increase the risk for features of autism in the offspring provide proof-of-principle for the role of environmental factors. Both the environmental and genetic factors can be very effectively modeled in mice.

• Certain neuropathologies that are relatively common in autism can be reproduced in rodent models.

Introduction

Animal models of many neurological diseases (Alzheimer's, Parkinson's, Huntington's, multiple sclerosis) have proven enormously useful for determining the roles of genes and environment, for understanding pathogenesis, and for testing potential therapeutic approaches. There is some skepticism, however, concerning models of psychiatric or mental illnesses (e.g., autism, schizophrenia, depression). After all, can cognitive abnormalities or language deficits be detected in animals? However, to give up on this approach would deny the application of powerful genetic and molecular tools to these critical illnesses. Moreover, animal models need not be perfect mimics of human diseases to be valuable. This is clear from the extensive and productive use of genetic mouse models for Huntington's and other neurodegenerative diseases, which do not exhibit the severe loss of particular types of neurons that characterize these disorders. The power of animal models is in the examination of key features of a disease, and the relevance of an animal model should be judged by how well it reflects one or more features of that disease, which may include genetics, neuropathology, behavior, etiology, electrophysiology, or molecular changes.

Autism is a particularly difficult case for animal studies because it has a heterogeneous behavioral phenotype, the susceptibility genes have not been firmly identified, and it does not have a pathognomonic histology that allows definitive diagnosis. Nonetheless, autism does have generally agreed upon features that are distinctive, such as a deficit of Purkinje cells (PCs), decreased hippocampal γ-aminobutyric acid (GABA-A) receptors, and elevated levels of brain-derived neurotrophic factor (BDNF) and platelet serotonin (5-HT) (Palmen et al., 2004; Pardo &

Eberhart, 2007; Amaral et al., 2008). There is also striking evidence for immune dysregulation in the autistic brain and cerebrospinal fl uid (Pardo et al., 2006; Arion et al., 2007; Chez et al., 2007; Pardo & Eberhart, 2007; Morgan et al., 2010). Moreover, some of the characteristic behavioral features of autism can be assayed in animals, such as neophobia, abnormal social interactions, stereotyped and repetitive motor behaviors, communication deficits (ultrasonic vocalizations; USVs), enhanced anxiety, abnormal pain sensitivity, disturbed sleep patterns, abnormal eye blink conditioning, and deficits in sensorimotor gating (prepulse inhibition; PPI) (Silverman et al., 2010).

Although autism has a strong genetic basis, it is not a monogenic disorder, and thus it is not possible to establish an immediately relevant genetic mouse model, as was done with Huntington's disease. Nonetheless, there are several genetic changes that do entail an elevated risk for autism, and mouse models of these changes share some features with the human disorder. There are also several human disorders caused by single gene mutations that display autistic features and mouse mutants of these mutations display behavior or neuropathology relevant to autism. In addition, models based on autism etiology are valuable, and there are several known environmental risk factors that are being successfully modeled in rodents. Finally, there are brain lesion models of interest. Therefore, even at this early stage of analysis, it is clear that various models can be used to study how particular genes influence certain autism endophenotypes, and how known environmental risk factors influence such endophenotypes. It will also be interesting to determine how a particular genotype influences the response to an environmental risk factor, and vice versa. There are currently very few examples of such gene-environment interactions in mouse models. This chapter discusses current genetic,

environmental risk factor and lesion models. Several other authors have reviewed various aspects of animal models related to autism (Murcia et al., 2005; Tordjman et al., 2007; Moy & Nadler, 2008).

Environmental manipulations

Thalidomide and valproic acid

Prenatal or early postnatal drug exposure can increase autism risk. Comorbidity of Moebius syndrome and autism support a correlation between autism and the use of the prostaglandin misoprostol, a drug historically administered for labor induction or abortion (Miller et al., 2004). Case studies of fetal alcohol syndrome also suggest that prenatal exposure to ethanol increases risk for autism (Nanson, 1992). Perhaps most clearly associated with autism, however, are the teratogens thalidomide (Stromland et al., 1994) and valproic acid (VPA; valproate) (Christianson et al., 1994). Not only can these drugs cause an array of birth defects, they also increase the incidence of autism when administered early in human pregnancy (Miyazaki et al., 2005).

The use of thalidomide led to the discovery of a window of vulnerability for the development of autism (Fig. 52.1). During the 1950s and 60s, thalidomide treatment of morning sickness resulted in thousands of offspring with severe malformations. Since the timing of drug exposure leads to specific types of craniofacial defects, the defects seen in the autistic offspring could be used to determine when these offspring were exposed. In this way, vulnerability to autism was pinpointed to days 20 to 24 of gestation, the time of neural tube closure and formation of motor nuclei and cranial nerves. Importantly, there is some evidence that idiopathic autism cases may also exhibit abnormalities in the

cranial nerve nuclei and other neuropathologies that originate during fetal brain development (Schneider & Przewlocki, 2005; Palmen et al., 2004).

A few laboratories have translated maternal thalidomide exposure into rodent models. Exposure of rats to thalidomide on embryonic day 9 (E9) yields adult offspring with hyperserotonemia in the plasma (as in autism), hippocampus, and frontal cortex (Narita et al., 2002), with altered distribution of serotonergic neurons in the raphe nuclei (Miyazaki et al., 2005). These offspring also display hyperactivity in the open field and decreased learning in the radial maze (Narita et al., 2010). Exposure on E15 inhibits vasculogenesis and alters cortical and hippocampal morphology (Fan et al., 2008). Furthermore, daily maternal injection of rats from E7 to E18 yields adult offspring with altered learning and memory as measured by increased errors and latency in the Cincinnati water maze (Vorhees et al., 2001). Clearly, much more could be done with this model to establish its relevance for autism.

Because VPA retains its teratogenicity in rodents, its administration during the time of neural tube closure has proven useful as a rodent model. VPA was fi rst introduced in the 1960s as an anticonvulsant and later as a mood-stabilizing drug for treatment of epilepsy and bipolar disorder (Markram et al., 2007). Like thalidomide, use of VPA during early human pregnancy significantly elevates the incidence of autism and the development of craniofacial defects in exposed offspring. Both case and epidemiological studies have confirmed the association between fetal valproate syndrome and autism (Hyman et al., 2006; Fan et al., 2008). Although women who are prescribed VPA for treatment of epilepsy often take the drug throughout pregnancy, it is unclear

whether brain regions other than the brain stem are vulnerable to VPA insult at later stages of development (Rinaldi et al., 2007a).

In animal studies, a single injection of VPA in a pregnant rat results in striking neuropathology and behavioral abnormalities. Offspring of rats injected with VPA show brain defects resembling those sometimes found in autism, including reduced number of motor nuclei and PCs (Schneider & Przewlocki, 2005), hyperserotonemia, and disorganized migration of 5-HT neurons in the dorsal raphe nuclei (Miyazaki et al., 2005). Fetuses from VPA-injected mothers display hypoplasia of the cortical plate, abnormal migration of dopaminergic and serotonergic neurons, and abnormal pons pathology (Kuwagata et al., 2009). VPA is a histone deacetylase inhibitor that is thought to impede neuronal differentiation and migration by interfering with the sonic hedgehog signaling pathway.

Interestingly, offspring of rats injected with VPA on E12.5 develop behavioral abnormalities that appear before puberty, a feature that distinguishes this model from behavioral changes seen in schizophrenia (Schneider & Przewlocki, 2005). VPA offspring display lower sensitivity to pain and higher sensitivity to nonpainful sensory stimuli, which parallels reported changes in endogenous opioid systems in some autistic patients. These offspring also exhibit impaired sensorimotor gating as measured by acoustic PPI, elevated anxiety as evidenced by decreased open field exploration, increased stereotypic/ repetitive activity, decreased social interaction, impaired reversal learning, and altered eyeblink conditioning patterns, and enhanced fear memory processing, all of which are consistent with results in autistic children (Stanton et al., 2007; Murawski et al., 2008; Markram et al., 2008; Dufour-Rainfray et al., 2010; Roullet

et al., 2010) (Table 52.1). A sexual dimorphism has been reported for some of these parameters, with abnormalities seen only in male offspring, which is also consistent with the very significant male bias in autism (Schneider et al., 2008). It will also be interesting to look for communication deficits (USVs) in this model.

Electrophysiological studies indicate that offspring of VPA treated mothers exhibit abnormal microcircuit connectivity in the neocortex and amygdala. Exposure to VPA results in overexpression of CaMKII and the NR2A and NR2B NMDA receptor subunits in the neocortex (Rinaldi et al., 2007b). These observations are consistent with observed increases in NMDA receptor-mediated synaptic transmission and enhanced postsynaptic long-term potentiation in neocortical pyramidal neurons. Adult VPA offspring also show increased connection probability of layer 5 pyramidal cells but decreased excitability and decreased putative pyramidal cell synaptic contacts (Rinaldi et al., 2008). These results relate to MRI studies showing impaired long-range functional connectivity in autistic individuals (Just et al., 2004).

Recordings from neurons in the lateral amygdaloid nucleus demonstrate hyper-reactivity to electrical stimulation, elevated long-term potentiation and impaired stimulus inhibition that may contribute to the deficient fear extinction and high anxiety seen in VPA offspring (Markram et al., 2008). These results suggest molecular and synaptic alterations in VPA mice that are relevant for the alterations in amygdala morphology observed in autism (Amaral et al., 2008). Dysfunction in the amygdala may contribute to the decreased social interaction and/or abnormal fear processing characteristic of autistic pathology (Markram et al., 2008). While deficits in social play and exploration have been

reported in VPA rodent offspring (Schneider et al., 2008), an important gap in the behavioral analysis of social interaction is in the analysis of social preference and USVs.

Although the mechanisms underlying the effects of prenatal VPA on fetal brain development are largely unknown, neural inflammation and gene regulation could be involved. Immunological alterations have been reported in offspring of VPA-treated mice, (Schneider et al., 2008; Bennett et al., 2000), and in vitro studies indicate that VPA promotes astrocyte proliferation, inhibits microglial and macrophage activation, and induces microglial apoptosis (Peng et al., 2005; Dragunow et al., 2006). This is consistent with the finding that VPA can regulate epigenetic modifi cations through three mechanisms: inhibiting histone deacetylases, enhancing histone acetylation, and promoting demethylase activity (Chen et al., 2007). The ability of VPA to alter HOX gene expression is particular interest, as HOXa1 is expressed during the time of neural tube closure and regulates development of the facial nucleus and superior olive (c.f., Finnell et al., 2002). Moreover, VPA treatment may actually promote neurogenesis of GABAergic neurons and facilitate neurite outgrowth (Dragunow et al., 2006). These neuroprotective effects occur after chronic VPA treatment rather than the acute exposure administered in the maternal VPA model, however (Hao et al., 2004; Ren et al., 2004).

Although maternal VPA and thalidomide exposure are responsible for only a small fraction of autism cases, the extremely high risk for autism in the offspring provides proof-of-principle for environmental influences on autism incidence. Moreover, the similarities in neuropathology and behavior between the rodent models and human autism support the utility of environment-based models for defining relevant pathways of developmental dysregulation. It will be important to extend the VPA model to mice

carrying genetic variants associated with increased risk for autism, which would provide a test of the gene x environment paradigm. Interestingly, prenatal VPA exposure has been linked to altered expression of neuroligin3, a genetic susceptibility factor for autism (Kolozsi et al., 2009).

Maternal Infection

Maternal infection is an environmental risk factor for the development of several neuropsychiatric disorders in the offspring. As is the case for schizophrenia (Patterson, 2007; Brown & Derkits, 2020), maternal viral infection is linked to higher incidence of autism by clinical, epidemiological, and case studies. Early evidence for this came from the 1964 rubella pandemic, in which the incidence of autistic features was increased more than 200-fold in the offspring of infected mothers (Chess, 1977). Case studies have linked autism to several other prenatal viral infections, including varicella, rubeola, and cytomegalovirus (Ciaranello & Ciaranello, 1995). Bacterial and protozoan infections have also been associated with autism (Nicolson et al., 2007; Bransfield et al., 2008). The most compelling evidence linking maternal infection with autism comes from a very large study utilizing the Danish Medical Birth Register. An examination of over 10,000 autism cases found a very significant association with maternal viral infection in the first trimester (Atladottir et al., 2010). In sum, the diversity of micro-organisms implicated in autism, along with the fact that several of these infections do not involve direct transmission into the fetus, suggests that the maternal immune response, rather than microbial pathogenesis, is responsible for increasing the risk for autism in the offspring (Fig. 52.2). Animal models of maternal infection further support the idea that maternal

immune activation (MIA) and the production of pro-inflammatory cytokines are what unite the various types of maternal infection as risk factors for autism. There are three primary rodent models for MIA: maternal influenza infection, poly(I:C) injection, and lipopolysaccharide (LPS) injection.

Pregnant mice intranasally infected with influenza yield offspring with behavioral and neuropathological abnormalities that parallel those seen in autism. Abnormal behaviors include heightened anxiety during open-field exploration, deficient PPI, decreased novel object exploration, and reduced social interaction (Shi et al., 2003). These offspring display spatially selective PC loss in lobules VI and VII (Fig. 52.3) (Shi et al., 2009), which is a common neuropathology in autism (Palmen et al., 2004; Amaral et al., 2008). There is also macrocephaly, delayed cerebellar granule cell migration, reduced Reelin immunoreactivity in the cortex, thinning of the neocortex and hippocampus, and altered expression of neuronal nitric oxide synthase and synaptosome-associated protein-25 (Fatemi et al., 2002; Shi et al., 2009). Infection on E16 or E18 causes altered expression of several genes associated with autism, white matter thinning in the corpus callosum, widespread brain atrophy, and altered levels of cerebellar 5-HT but not dopamine (Fatemi et al., 2008, 2009; Winter et al., 2008).

Because maternal influenza infection is largely confined to the respiratory tract, it is unlikely that these neurological defects are caused by direct viral infection of the fetus. There are, however, conflicting reports as to whether viral mRNA or protein is present in fetal tissues (Aronsson et al., 2002; Shi et al., 2005). Nonetheless, the fact that stimulating the maternal immune system with poly(I:C) (mimicking viral infection) and LPS (mimicking bacterial infection) causes neuropathogical and behavioral defects in the

offspring similar to those seen with maternal influenza infection supports the idea that MIA is the causative event, as no pathogen is required. Poly(I:C) is a synthetic, double-stranded RNA that generates an antiviral immune response in the absence of virus. Depending on the dosage, mode of injection (intraperitoneal or intravenous) and timing of maternal poly(I:C) administration, offspring display deficits in PPI, latent inhibition, open field exploration, working memory, social interaction and USVs, while reversal learning and amphetamine-induced locomotion are enhanced (Shi et al., 2003; Zuckerman et al., 2003, 2005; Lee et al., 2007; Meyer et al., 2007; Smith et al., 2007; Winter et al., 2009; Malkova & Patterson, 2010). A single poly(I:C) injection also causes histopathological changes similar to those seen in autism, including increased GABAA receptor, spatially-restricted reduction in PCs, and delayed myelination, and decreased cortical neurogenesis (Nyffeler et al., 2006; Shi et al., 2009; Makinodan et al, 2008; De Miranda et al., 2010). A cardinal pathology in schizophrenia, enlarged lateral ventricles, is also observed (Li et al., 2009; Piontkewitz, Assaf, & Weiner, 2009). In addition, there is evidence of physiological abnormalities in the hippocampus. In slices from adult offspring of poly(I:C)-treated mothers, oscillations in CA1 are less rhythmic than in controls, and CA1 pyramidal neurons display reduced frequency and increased amplitude of miniature excitatory postsynaptic currents. Differing results have been reported regarding a deficit in long term potentiation (Ito et al, 2010; Oh-Nishi et al., 2010). Interestingly, the specific component of the temporoammonic pathway that mediates object-related information displays significantly increased sensitivity to dopamine (Lowe et al., 2009; Ito et al., 2010). There are a few studies describing abnormal dopamine levels in autism (Previc, 2007), and a variety of changes in dopamine are found in the

maternal poly(I:C) model (Zuckerman et al., 2003; Ozawa et al., 2006; Meyer et al., 2008a). However, whether dysregulation of the dopaminergic system is an important feature of autism is unknown. Dopamine pathology is very important in schizophrenia, where maternal infection is also a risk factor. Another finding consistent with both schizophrenia and autism is a disruption in long-range synchrony of neuronal firing. Adult MIA offspring display significant reduction in medial prefrontal cortex-hippocampal EEG coherence (Dickerson, Wolff & Bilkey, 2010).

Further supporting the role of MIA in altering fetal brain development is the use of maternal LPS injection to simulate bacterial infection. Although poly(I:C) and LPS act through different toll-like receptors, their effects on the behavior and brain pathology in offspring often overlap. For example, a single injection of LPS in a pregnant rat yields offspring with elevated anxiety, aberrant social behavior, reduced play behavior and USVs, reduced PPI, enhanced amphetamine-induced locomotion, and abnormal learning and memory (Borrell et al., 2002; Fortier et al., 2004; Golan et al., 2005; Hava et al., 2006; Basta-Kaim et al., 2010; Baharnoori et al., 2010; Hao et al., 2010; Kirsten et al., 2010), many of which parallel behaviors seen in autism. There is also evidence of hyperactivity in the hypothalamus-pituitary-adrenal axis in the LPS offspring, and some of the abnormal behaviors can be reversed by anti-psychotic drug treatment (Basta-Kaim et al., 2010), as is the case for the poly(I:C) offspring. Recall that maternal infection is a risk factor for schizophrenia as well as autism.

Histological findings include smaller, more densely packed neurons in the hippocampus, increased numbers of pyknotic cells in the cortex, fewer tyrosine hydroxylase-positive (TH+) neurons in the substantia nigra, and increased TH+ cells in

the nucleus accumbens (Golan et al., 2005; Ling et al., 2004; Borrell et al., 2002). Further studies indicate that changes in dendritic length, dendritic branching, spine structure, and spine density in the medial prefrontal cortex and hippocampus, suggesting dysregulated neuronal connections formed during embryogenesis (Baharnoori et al., 2008). Some of these effects, including increased cell density and limited dendritic arbors in the hippocampus, have been found in MRI and post mortem brain studies in autism (Amaral et al., 2008). Electrophysiological recordings reveal reduced synaptic input to CA1 of the hippocampus, heightened excitability of pyramidal neurons, enhanced postsynaptic glutamatergic response, and impaired NMDA-induced synaptic plasticity (Lowe et al., 2008; Lante et al., 2008). Interestingly, many of these effects are prevented by pretreatment of pregnant rats with N-acetyl-cysteine (Lante et al., 2008), which increases calcium influx when binding to glutamate receptors in combination with the transmitter. Brain imaging studies of the hippocampus and of particular neurotransmitter systems in autism have yielded inconsistent results, so no definite statement can be made about their exact roles in autism (Palmen et al., 2004).

In addition to the behavioral deficits and neuropathology, the MIA models also share with autism dysregulation of immune status in the brain. Post mortem brain and cerebrospinal fluid samples from autistic individuals exhibit marked astrogliosis, microglial activation, dysregulation of immune-related genes, and high levels of pro-inflammatory cytokines and chemokines (Vargas et al., 2005; Garbettet al., 2008; Chez et al., 2007; Tetreault et al., 2009; Patterson, 2009). Although LPS itself does not cross the placental barrier, maternal LPS injections yield offspring with MHC II induction along with increased GFAP and microglial staining in various adult (Borrell 2002; Ling et al.,

2004) and fetal (Paintlia et al., 2004) brain regions. While several cytokines are elevated in the placenta and amniotic fluid after MIA, mRNA transcripts for a number of cytokines are also elevated in the fetal brain following maternal LPS or poly(I:C) (Urakubo et al., 2001; Cai et al., 2000; Paintlia et al., 2004; Liverman et al., 2006; Golan et al., 2005; Meyer et al., 2008b; Elovitz et al., 2006; E. Hsiao & P.H. Patterson, unpublished). The importance of cytokines as soluble mediators of the effects of MIA on fetal brain development was demonstrated using cytokine knockout (KO) mice and mice injected with recombinant cytokines or cytokine-neutralizing antibodies. Interleukin (IL)-6 is necessary and sufficient for mediating the effects of MIA on the development of neurological, behavioral, and transcriptional changes in poly(I:C)-exposed offspring (Fig. 52.2; Samuelsson et al., 2006; Smith et al., 2007). In a converse approach, overexpression of the anti-inflammatory cytokine IL-10 suppresses the effects of maternal poly(I:C) on the fetus (Meyer et al., 2008c). Perturbation of IL-10, IL-1, or TNFa can also significantly influence the outcome of MIA in the LPS MIA model (Girard et al., 2010; reviewed in Patterson, 2011).

How cytokines induced by MIA alter the course of fetal brain development is largely unknown. The most obvious possibility is by direct action on the developing brain, as both cytokines and chemokines are key modulators of astrogliosis, neurogenesis, microglial activation, and synaptic pruning (Bauer et al., 2007; Deverman & Patterson, 2009), and some maternal cytokines have been reported to cross the placenta (Dahlgren et al., 2006; Zaretsky et al., 2004). A second possibility is that MIA alters the endocrine function and/or the immunological state of the placenta. In fact, poly(I:C) MIA increases maternally-derived IL-6 protein as well as IL-6 mRNA in the placenta. Such placentas

exhibit increases in CD69+ decidual macrophages, granulocytes and uterine NK cells, indicating elevated early immune activation. Moreover, maternally-derived IL-6 mediates activation of the JAK/STAT3 pathway in the placenta, which parallels an IL-6-dependent disruption of the growth hormone-insulin-like growth factor axis (E. Hsiao & P. H. Patterson, unpublished data). Such endocrine changes could affect the development of the fetal brain and immune system, with permanent consequences. It is notable that a greater occurrence of placental trophoblast inclusions is observed in placental tissue from births of children who develop autism spectrum disorder compared to non-ASD controls (Anderson et al., 2007). Moreover, chorioamnionitis and other obstetric complications are significantly associated with socialization and communication deficitis in autistic infants (Limperopoulos, 2008).

In this context, it is of interest that several studies have reported that the sera of some mothers of autistic children contain antibodies that bind fetal human, monkey, or rat brain antigens (Zimmerman et al., 2007; Braunschweig et al., 2008; Martin et al., 2008). Most relevant to this review is the further finding that injection of such maternal sera into pregnant mice (Dalton et al., 2003) or purified maternal IgG into pregnant Rhesus monkeys (Martin et al., 2008) yields offspring with several behavioral abnormalities, including hyperactivity and stereotopies in the case of the monkeys. That something is different about the immune system of in the mothers of autistic offspring is further supported by the observation that these mothers are more likely to have a history of autoimmune disease or asthma (e.g., Altadottir et al., 2009). There is also evidence that the peripheral immune system of autistic subjects is abnormal (Pardo & Eberhart, 2007; Enstrom, Van de Water, & Ashwood, 2009). In that light it is interesting that, compared

to controls, CD4+ T cells from the spleen and mesenteric lymph nodes of adult mouse MIA offspring display significantly elevated IL-6 and IL-17 responses to in vitro stimulation (Hsiao et al., 2010; Mandal et al., 2010). Furthermore, adult MIA offspring display reduced T cell responses to CNS-specific antigens, despite elevated proliferation of nonspecific T cells (Cardon et al., 2009).

Although it is commonly stated that autism can result from an environmental stimulus acting on a susceptible genetic background, there is little support for this hypothesis thus far. Thus, it is of interest that mice heterozygous for the tuberous sclerosis 2 (TSC2) gene display a social interaction deficit only when they are born to mothers treated with poly(I:C) (Ehninger et al., 2010). That is, this deficit is most severe when the MIA environmental risk factor is combined with a genetic defect that, in humans, also carries a very high risk for ASD. In addition, there is an excess of TSC-ASD individuals born during the peak influenza season, an association that is not seen for TSC individuals not displaying ASD symptoms (Ehninger et al., 2010).

Postnatal Vaccination

Although there is currently no convincing evidence that postnatal vaccination is a cause of autism, the occasional coincidence in the timing of routine childhood immunizations with the appearance of autistic symptoms continues to fuel public concern. The measles-mumps-rubella (MMR) vaccine is of particular interest because of the use of live, attenuated virus, but there are currently no rodent models for the effects of MMR on neural development. Moreover, many epidemiological studies have failed to substantiate a connection between MMR vaccination and autism (DeStefano, 2007).

There has been investigation of the effects of thimerosal-containing vaccines (TCVs) on neurodevelopment in rodents. Increased mercury burden from this sodium ethylmercurithiosalicylate preservative is of concern because of known neurotoxic properties of methylmercurials. One study reported that immunogenetic factors can render mice susceptible to thimerosal-induced neurotoxicity (Hornig et al., 2004). The autoimmune-prone SJL/J (H-2s) strain developed neuropathology and abnormal behavior. However, a recent study failed to replicate the histological and behavioral results (Berman et al., 2008). This latter paper is consistent with several epidemiological studies failing to support a link between thimerosal and autism (Andrews et al., 2004; Schechter & Grether, 2008; Gerber & Offit, 2008 Price et al., 2010).

Terbutaline

The b2 adrenergic receptor is expressed early in fetal brain development, and its activation affects cell proliferation and differentiation. Terbutaline is a selective b2 adrenergic receptor agonist that is used to relax uterine smooth muscle to prevent premature labor and birth. A study of dizygotic twins found an increased rate of concordance for autism if the mother was given terbutaline for 2 weeks (Conners et al., 2005). Further implicating the b2 adrenergic receptor is the finding that certain functional variants of this gene are associated with increased risk for autism (Cheslack-Postava et al., 2007). In modeling this risk factor in rats, neonates are given subcutaneous injections of terbutaline daily, from postnatal days 2 through 5, a time meant to mimic the stage of human brain development at which the drug is given. A significant deficit in PCs was found, but no mention of any spatial restriction of this change was made.

Histological changes were reported in the hippocampus and somatosensory cortex as well, including microglial activation in cortex and cerebellum (Rhodes et al., 2004; Zerrate et al., 2007). Also of interest in terms of autism is a finding of increased 5-HT turnover (Slotkin & Seidler, 2007). Behavioral analysis of this model is somewhat disappointing thus far, with female-specific hyperactivity and no change in PPI (Zerrate et al., 2007).

Genetic Manipulations

X-Linked and Autosomal Lesions

Fmr1 Knockout Mice

Fragile X Syndrome (FXS) is an X-linked condition that is the leading genetic cause of mental retardation (Hatton et al., 2006). It is caused by the loss of expression of FMRP, an mRNA-binding protein that is highly expressed in hippocampal and cortical synapses, where it regulates translation of its target mRNAs and thus plays a key role in protein synthesis-dependent functions (Bassell & Warren, 2008). It is estimated that 90% of FXS patients present some autistic-like behaviors, and that 15% to 33% meet the full diagnostic criteria of autism (Cohen et al., 1988; Bailey et al., 1998). Overall, FXS accounts for about 5% of the autistic population (Li et al., 1993). Fmr1 KO mice display some anatomical features of FXS, such as macro-orchidism and abnormal dendritic development and morphology (The Dutch-Belgian Fragile X Consortium, 1994), and spines are altered in idiopathic autism (Zoghbi, 2003). These mice also display some core behavioral features relevant to autism, including impaired social interaction

(McNaughton et al., 2008) and repetitive behaviors. Whether they display learning and memory deficits is unclear (Dobkin et al., 2000; Frankland et al., 2004), and whether they show other autism-related symptoms such as anxiety and hyperactivity depends on the genetic background (Bernardet & Crusio, 2006). The observations that both cognitive performance and behaviors relevant to autistic traits are affected by genetic background is of interest given the genetic variability in humans and the phenotypic heterogeneity in FXS. Although overall resemblance to autism is partial, the presence of two core features of autism indicates that molecular investigation of Fmr1 KO mice may further our understanding of the genetic etiology of FXS and autistic traits.

The signaling pathways for metabotropic glutamate receptor 5 (mGluR-5) and PAK, a kinase involved in actin remodeling and regulation of synapse structure, represent two plausible therapeutic targets for FXS and autism. A 50% reduction of mGluR-5 expression in Fmr1 KO mice normalizes dendrite morphology, seizure susceptibility, and inhibitory avoidance extinction (Dolen et al., 2007) (Table 52.2). This supports the mGluR theory, which posits that upregulation of group 1 mGluR leads to exaggerated protein synthesis-dependent functions, such as long-term depression, and therefore underlies the neuropathology and behavioral traits associated with FXS (Bear, Huber, & Warren, 2004). Interestingly, postnatal inhibition of PAK in the forebrain of Fmr1 KO mice normalizes dendrite morphology and restores locomotion, repetitive behavior, and anxiety (Hayashi et al., 2007). In addition, in a Drosophila model, treatment with mGluR antagonists or protein synthesis inhibitors in adulthood can partially restore deficits in courtship behavior and improve memory (McBride et al., 2005; Bolduc et al., 2008). Two small, open label human trials based on these findings yielded promising results (Berry-

Kravis et al., 2008, 2009; Paribello et al., 2010), supporting the use of FXS animal models for preclinical purposes.

Methyl-CpG-Binding Protein-Null, Mutant, and Overexpressing Mice

Rett syndrome is another X-linked disorder that causes mental retardation, primarily affecting females. It is estimated that 95% of Rett syndrome cases are caused by mutations in the methyl-CpG-binding protein (MECP2) gene (Chahrour & Zoghbi, 2007), leading to deficiency in this global transcriptional regulator, whose targets include BDNF. During the regression phase of the disease, affected girls display autistic-like behaviors, such as stereotypies as well as reduced social contact and communication. Association between MECP2 variants and autism have also been reported (Loat et al., 2008). Both MECP2-null mice, and mice in which MECP2 is deleted in mature neurons only, exhibit a neurological phenotype consistent with Rett, including hypoactivity, ataxic gait, tremor, limb-clasping, and reduced brain size with smaller neuronal cell bodies in cortex and hippocampus (Chen et al., 2001; Guy et al., 2001). BDNF levels are also reduced in comparison to wild-type (WT) animals, and deletion or over-expression of Bdnf in the Mecp2 mutant brain either accelerates or delays the onset of the symptoms, suggesting a functional interaction between MeCP2 and BDNF in vivo (Chang et al., 2006). Male mice that carry the truncating mutation, Mecp2308/y, a common variant observed in Rett patients, display a milder Rett-like phenotype (Shahbazian et al., 2002). Increased synaptic transmission and impaired LTP induction is observed in the mutant mice, whereas spine morphology, BDNF levels, and synaptic biochemical composition are not altered. Behavioral deficits in these mice include enhanced anxiety in the open

field, reduced nest-building, and aberrant social interactions. Genetic background modifies performance in the Morris water maze, latent inhibition, and long-term memory tasks (Moretti et al., 2005; Moretti et al., 2006). Mice over-expressing MeCP2 also develop a progressive neurological disorder with, surprisingly, an enhancement in synaptic plasticity, motor and contextual learning skills between age 10 and 20 weeks, and, at an older age, hypoactivity, seizures, and abnormal forelimb-clasping, all of which are reminiscent of human Rett syndrome (Collins et al., 2004). These results with the various MeCP2 mouse models indicate that this gene must be tightly regulated under normal conditions. These mice should aid in the search for genes that are regulated by MeCP2 (Chahrour et al., 2008) and possibly the various behavioral abnormalities. These mice are also providing reason for optimism regarding the testing of potential treatments for Rett Syndrome. In a conditional KO model, it was shown that restoring MeCP2 expression in immature or even in mature mice results in reversal of the disease phenotype, as measured by behavioral and electrophysiological tests (Guy et al., 2007). Thus, despite the fact that MeCP2 function was disrupted during fetal and postnatal development, the disease symptoms can be reversed. In one test of a potential treatment, administration of an active peptide fragment of insulin-like growth factor 1 to MeCP2 mutant mice extends life span, improves locomotor, heart and breathing functions, and stabilizes a measure of cortical plasticity (Tropea et al., 2009). Such results provide proof-of-principle that these mice can be used to screen candidate treatments of autism-related disorders.

Angelman and Prader-Willi Syndromes

Loss of function of maternal or paternal genes in the imprinted chromosomal region 15q11-q13 causes Angelman Syndrome and Prader-Willi Syndrome (PWS), respectively. Although clinically distinct, both syndromes are behavioral disorders presenting with some autistic traits as well as other diverse symptoms (Veltman et al., 2005). Linkage studies have also associated the 15q11-q13 locus with autism, and maternal duplications of this region account for rare cases of autism (Wassink & Piven, 2000). 70% of Angelman Syndrome patients carry large maternal deletions of 15q11-q13, and display a severe phenotype; yet, mutations in a single gene, UBE3A, are sufficient to cause major clinical manifestations of the syndrome. By contrast, PWS is clearly a multigenic syndrome involving 10 imprinted genes, whose individual significance in the etiology of the disorder is not yet fully clarified (Nicholls & Knepper, 2001). UBE3A encodes E6-AP, an enzyme that has ubiquitin protein ligase and transcriptional coactivator activities (Nawaz et al., 1999). Two different KO mouse strains with maternally inherited mutations in Ube3a display a phenotype consistent with human Angelman Syndrome: motor dysfunction, propensity for seizures, defective learning and memory, and abnormal electroencephalograms (Jiang et al., 1998; Miura et al., 2002). These mice also display deficits in hippocampal LTP and decreased hippocampal CaMKII activity, which may contribute to learning problems in Angelman Syndrome (Weeber et al., 2003). When crossed to mice carrying a mutation in CaMKII that prevents its autophosphorylation, the double mutants no longer exhibit the Angelman Syndrome phenotype (van Woerden et al., 2007). This suggests that increased inhibitory autophosphorylation may provide a molecular basis for deficits in LTP, motor coordination, and seizure propensity. Studies of another Angelman Syndrome mouse

model, the Ube3aYFP knock-in (KI) reporter mouse, reveal that E6-AP is found in synapses and the nucleus (Dindot et al., 2008). These mice display decreased spine density, an interesting finding because altered spine morphology is observed in Rett and FXS patients, as well as in Fmr1 KO mice (Kaufmann & Moser, 2000). Thus, the neuropathology in this KI suggests that E6-AP could play a role in spine development and synaptic plasticity. It is relevant that loss of UBE3A activity or its overexpression in Drosophila reduces dendritic branching and affects dendrite morphogenesis (Lu et al., 2009). It remains to be determined whether similar neuropathology is present in the KO mouse lines and Angelman Syndrome patients and whether it contributes to cognitive dysfunction and behavioral abnormalities.

More recently, mutant mice carrying a large maternal deletion from Ube3a to Gabrb3 were generated (Jiang et al. 2010). Similar to the Ube3a KO mice, these mutants display increased spontaneous seizure activity, abnormal electroencephalograms, as well as impairments in learning and memory. Additional behavioral tests reveal that they display anxiety traits in the light-dark box, but no difference in pain sensitivity or in PPI. Mutant newborn pups emit more USVs than control mice. This latter observation is of interest, since Angelman syndrome patients show a happy disposition that is currently interpreted as increased signaling behavior. Relevance to autism is also possible, as increased USVs have been reported in Mecp2 mutant and BTBR pups. However, one would expect to see fewer USVs in an autism model, given the deficits in communication in ASD. Comparative studies of the various mouse models of Angelman Syndrome should yield new insights into the contribution of additional, maternally imprinted genes of this region, or biallelically expressed genes such as Gabrb3 or Atp10a. Mouse models

for PWS with deletion of the corresponding murine imprinted locus have been generated, but early postnatal lethality has precluded behavioral characterization (Yang et al., 1998). Among the mice engineered to carry a mutation in one candidate gene of the imprinted region, Necdin (Ndn), paternally-deficient mice display some behavioral traits reminiscent of PWS, such as skin-scraping, improved performance in Morris water maze, and reduced numbers of hypothalamic oxytocin and LHRH-producing neurons (Muscatelli et al., 2000). Serotonergic alterations are also observed in these mice and are linked to respiratory deficiency (Zanella et al., 2008). All these findings might be relevant to autism, because repetitive self-injury, enhanced visual-spatial skills, oxytocin abnormalities, and alterations in serotonin levels have been described in autism. As with Angelman Syndrome mice, Necdin mutant mice require further behavioral characterization.

Pten Mutant Mice

Phosphatase and tensin homolog on chromosome 10, PTEN, is a tumor suppressor that negatively regulates phosphatidylinositol 3-kinase PI3K/Akt signaling, a pathway that promotes cell growth, proliferation, and survival. Germline mutations in PTEN cause Cowden and Bannayan–Riley–Ruvalcaba syndromes (CS and BRRS, respectively). CS and BRRS are characterized by benign and malignant tumors in multiple organs as well as brain disorders such as macrocephaly, mental retardation, and seizure. Association between these syndromes—particularly CS—and autism has occasionally been reported (Zori et al., 1998; Goffin et al., 2001; Pilarski & Eng, 2004). Moreover, genetic screening identified PTEN mutations in a subset of autistic individuals

who display macrocephaly (Butler et al., 2005; Buxbaum et al., 2007), an anatomical anomaly also present in 15% to 20% of autistic patients (Lainhart et al., 2006). Of particular interest is the mouse strain Nse-cre-PtenloxP/loxP, in which a Pten deletion is restricted to differentiated neurons in the cerebral cortex and hippocampus (Kwon et al., 2006) These mutants develop forebrain macrocephaly resulting from neuronal hypertrophy in the cortex and hippocampus. In addition, analysis of the hippocampus shows increased dendritic and axonal growth, ectopic positioning of axons and dendrites of granule neurons and elevated synapse number.

Abnormalities in Pten-deleted neurons correlate with enhanced levels of phosphorylated Akt and its downstream effectors, mTOR and S6. Components of the mTOR/S6K/S6 pathway are present in dendrites, where they are involved in the regulation of protein synthesis. Protein synthesis in dendrites is believed to modulate synapse morphology and function and thus is involved in synapse plasticity. These mice display behaviors reminiscent of autism, such as deficits in social interaction, exaggerated responses to sensory stimuli, decreased PPI (but only at one prepulse stimulus intensity), anxiety-like behavior in the open field, and learning deficits in the Morris water maze. However, no impairments in fear conditioning, elevated plus maze, or motor activity are observed. Increased spine density and social deficits are also observed in the Fmr1 KO. In this context, it is relevant that ribosomal S6 kinase (S6K1), a component of the mTOR/PI3K signaling cascade, was recently identified as a major FMRP kinase (Narayanan et al., 2008). Because the phosphorylation status of FMRP may govern translational regulation of its target mRNAs, upstream modulators of the mTOR/PI3K pathway such as PTEN may modulate synaptic function by affecting FMRP

phosphorylation status (Bassell & Warren, 2008). Thus, FMRP phosphorylation may be affected in CS, BRRS, and also tuberous sclerosis (TSC), another human disorder associated with autism, caused by mutations in the TSC1/2 complex. Indeed, hamartin and tuberin, the gene products of TSC1 and TSC2, can inhibit mTOR (Yates, 2006). Moreover, mTOR signaling is dysregulated in the FMRP-deficient mouse (Sharma et al., 2010). Taken together, these data support the hypothesis that synaptic alteration may underlie autistic-like behaviors (Zoghbi, 2003) and highlight the mTOR pathway as a key regulator of synaptic function. In addition, as in the case of the MeCP2 mice, the Tsc2+/mouse model responds to treatment in adulthood. Brief administration of the mTOR inhibitor rapamycin rescues synaptic plasticity and the behavioral deficits in the TSC model (Ehninger et al., 2009). Moreover, early phase clinical trials suggest that cognitive features of TSC may be reversible in adult humans (De Vries, 2010).

Autism Candidate Genes

Neuroligins 3 and 4

Recent findings in autism genetics have revealed several, rare causal variants that are associated with ASD (Betancur, Sakurai & Buxbaum, 2009). Neuroligins (NLGNs) constitute a family of transmembrane postsynaptic proteins, which, together with their presynaptic and intracellular binding partners, the β-neurexins and SHANK3, respectively, play a key role in synaptic maturation and transmission. NLGN-3 and -4 were identified in two X-chromosome loci previously associated with autism spectrum disorders (ASDs) (Jamain et al., 2003). Thus far, one missense mutation in NLGN-3 and four missense and two nonsense mutations in NLGN-4 have been identified in a very

small number of individuals with ASDs (Jamain et al., 2003; Laumonnier et al., 2004; Yan et al., 2005), supporting the hypothesis that synaptic dysfunction is important in ASDs. Mutations in neurexin and SHANK3 are also found in ASD probands, but whether they are involved in ASD etiology is controversial (Sudhof, 2008). There is currently no KO for Shank3, but a KO for Shank1, the closest relative to Shank3, was recently created. These mutants display morphological alterations in hippocampal neurons that are associated with a reduction in basal synaptic transmission, but no change in several other electrophysiological parameters (LTP, LTD, and L-LTP). Behaviorally, Shank1 KO mice exhibit increased anxiety, impaired contextual fear memory, and, surprisingly, enhanced performance in a spatial learning task but impaired memory retention of that task (Hung et al., 2008). Additional behavioral tasks relevant to the three core symptoms have yet to be reported.

Results with NLGN-3 and -4 mutant mice confirm the functional significance of NLGNs in synaptic function. A NLGN-3 KI mouse was engineered with a point mutation in the endogenous mouse gene that is identical to the relevant human NLGN3 gene (Tabuchi et al., 2007). These mice display increased inhibitory synaptic transmission without a change in excitatory transmission, a phenotype not observed in NLGN-3 KO mice, emphasizing the disparity between missense and nonsense mutations. It will be ofinterest to characterize the behavior of the KO mice to check for differential phenotypes. The augmentation in inhibitory synaptic transmission in the NLGN-3 KI mice is accompanied by a deficit in social interaction and, as observed in the Shank1 KO, enhanced spatial learning ability. These results are surprising because (1) a loss, rather than a gain of inhibition in different neural systems was hypothesized to contribute to

ASDs (Hussman, 2001; Rubenstein & Merzenich, 2003), and (2) the individuals identified with mutations in NLGN-3 and -4 do not exhibit potentiated learning skills. The latter observations are consistent with a report of minimal aberrant behaviors in the NLGN-3 KI mice (Chadman et al., 2008). Nevertheless, the results suggesting that a disequilibrium between excitatory and inhibitory synapses can affect social behavior (Sudhof, 2008) and that decreasing inhibitory transmission may be an effective therapy in some autism patients are worth pursuing. In fact, administration of the NMDA receptor partial co-agonist D-cycloserine can rescue the excessive grooming behavior in adult NLGN-1 KO mice (Blundell et al., 2010).

Unlike humans, the rodent Nlgn4 gene localizes to a still unknown autosome. Although there is only a 57% homology between the two species, the protein is found in synapses in both. Although NLGN-4 KO mice display abnormalities in two of the three core autistic symptoms, reciprocal social interaction, and impaired communication, as approximated by measuring USVs, they do not display repetitive behavior or impairments in some of the other autism symptoms such as sensory ability, sensorimotor gating, locomotion, exploratory activity, anxiety, or learning and memory (Jamain et al., 2008). These observations are consistent with those seen in patients with the NLGN-4 mutation, who do not show these comorbid features. MRI analysis of the brains of NLGN-4 KO mice show a slight reduction in size of the total brain, cerebellum, and brain stem, and some of these neuroanatomical changes are reminiscent of autism.

To summarize, several NLGN models exhibit strong construct validity with the rare human mutations associated with human ASDs. Moreover, the face validity of the

NLGN-4 KO mice is fairly good at the behavioral level, but much remains to be done on its neuropathology.

CNTNAP2

One of the most validated susceptibility genes is contactin associated protein-like2 (CNTNAP2). This gene encodes a member of the neuronal neurexin superfamily that is involved in neuron-glial interactions and is very likely to be important in brain development (Abrahams et al., 2008b). An intriguing feature of CNTNP2 is its enriched expression in circuits in the human cortex that are important for language development. Moreover, its expression is enriched in song nuclei important for vocal learning in the zebra finch, and feature is male-specific, as is the song behavior (Panaitof et al., 2010). In addition, CNTNP2 polymorphisms are associated with language disorders, and the expression of this gene can be regulated by FOXP2, a transcription factor that, when mutated, can cause language and speech disorders (Vernes et al., 2008). In light of these associations, it is important that recent study of the Cntnap2 KO mouse reveals a deficit in USVs. Moreover, these mice display the other core features of autism, repetitive behavior and a social interaction deficit. They also exhibit several other features of ASD: seizures, mild cortical laminar disorganization and hyperactivity (D. H. Geschwind, personal communication).

En-2

ENGRAILED-1 (En-1) and -2 encode transcription factors expressed during embryonic and postnatal stages that regulate the development of the cerebellum. En-2

localizes in proximity to an autism susceptibility locus on chromosome 7 (Liu et al., 2001; Alarcon et al., 2002), and genetic variations in En-2 have also been reported to associate with ASDs (Petit et al., 1995; Gharani et al., 2004; Benayed et al., 2005; Wang et al., 2008; Yang et al., 2008), although one report could not replicate such association (Zhong et al., 2003). Although mice homozygous for a mutation in En-1 lack a cerebellum and die shortly after birth (Wurst, Auerbach, & Joyner, 1994), En-2 KO mice are viable and display some cerebellar pathologies resembling those reported in the brains of some autistic individuals, such as a decreased PC number, hypoplasia, and abnormal foliation (Kuemerle et al., 1997; Amaral, Schumann, & Nordahl, 2008). The juvenile KO mice display reduced social and play behaviors, and abnormal social behavior and repetitive self-grooming as adults (Cheh et al., 2006). In addition, although En2 KO mice display normal locomotor activity in the open field, motor deficits are observed in specific tasks such as mid-air righting, hanging-wire grip strength, and rotorod. Learning and memory impairments are also evident in the water maze and modified open field with objects. At the neurochemical level, mutant mice exhibit increased cerebellar serotonin compared to controls but no alteration in dopamine levels in hippocampus, striatum, and frontal cortex or cerebellum. Thus, En2 KO mice display face validity for autism, except for the motor deficits, which can interfere with some behavioral tests. It will be of interest to examine USVs in this model.

Serotonin

Several lines of evidence indicate that changes in serotonin signaling may contribute to autism pathogenesis. Serotonin levels in platelets are elevated in autistic

patients (Cook & Leventhal, 1996), and numerous polymorphisms in genes implicated in 5-HT signaling or metabolism have been reported in autism, including the serotonin-transporter gene SLC6A4 (SERT), monoamine oxidase A (MAOA), tryptophan 2,3 dioxygenase gene, and two serotonin receptors, 5-HT2A (HTR2A) and 5-HT7 (HTR7). Pharmacological modulation of the serotonin system using the 5-HT receptor antagonist risperidone improves ritualistic behavior and irritability of autistic children and, similarly, selective 5-HT reuptake inhibitors ameliorate repetitive thoughts and behaviors as well as mood disturbances. Conversely, depletion of tryptophan, a serotonin precursor, aggravates autistic symptoms (Hollander et al., 2005). Abnormalities in brain serotonin synthesis at different ages, as well as cortical asymmetries in serotonin synthesis, have been reported in children with autism (Chugani et al., 1997; Chugani et al., 1999). These alterations could be linked to abnormalities in cortical minicolumn organization in autism (Casanova & Tillquist, 2008). Serotonin signaling modulates various aspects of preand postnatal brain development (Gaspar, Cases, & Maroteaux, 2003), and some mouse lines with disruption in the 5-HT system show neuropathology consistent with those observed in autism. Behavioral changes observed in these mutants relate to mood, aggression, anxiety, depression, seizure, and learning and memory, all of which are relevant to autism.

A mouse line in which serotonin signaling is impaired is the Dhcr7 mutant. DHCR7 (7-dehydrocholesterol reductase) is an enzyme required for the biosynthesis of cholesterol, and mice lacking functional DHCR7 display an increase in the area and intensity of serotonin immunoreactivity in the embryonic hindbrain (Waage-Baudet et al., 2003). Unfortunately, Dhcr7 KO mice die shortly after birth, precluding behavioral

studies. In humans, DHCR7 deficiency causes the Smith–Lemli–Opitz Syndrome (SLOS), a disease characterized by dysmorphic facial features, mental retardation, and limb defects (Yu & Patel, 2005). Approximately 50% of patients with SLOS are also diagnosed with autism (Tierney et al., 2001). Levels of cholesterol are decreased in some idiopathic autistic children (Tierney et al., 2006), and cholesterol dietary supplementation improves autistic-like behavior of patients with SLOS (Aneja & Tierney, 2008). Presently, the mechanisms by which cholesterol deficiency affects serotonin pathways are not fully elucidated, but it is known that cholesterol can modulate the functional activity of MAO (Caramona et al., 1996) and SERT (Scanlon, Williams, & Schloss, 2001). Investigation of Dhcr7 heterozygous mice or development of conditional mutants could further the understanding of the role of cholesterol in autism.

BDNF-deficient mice also display alterations in the serotonin system. Signaling mediated by BDNF and its receptor tyrosine kinase (TrkB) is crucial for serotonergic neuronal development, as well as a wide variety of other neuronal functions. Variants in the BDNF gene have been associated with autism (Nishimura et al., 2007), and post mortem analysis of brains from autistic adults show enhanced levels of BDNF (Perry et al., 2001), whereas blood and serum levels in autism are controversial (cf. Croen et al., 2008). Various BDNF or TrkB mutant mouse lines are available, including heterozygous null mice (homozygous KOs are not viable), conditional, and inducible BDNF KO mice. Presence and severity of behavioral alterations in these mice depends on the stage at which BDNF is depleted, the brain regions targeted for BDNF deficiency, and the gender of the mutants (cf. Monteggia et al., 2004). Autistic-like behavioral impairments commonly reported are heightened aggression, hyperactivity, depression-like traits, and,

in some instances, altered locomotor activity. Hyperphagia is reported in some BDNF mutant lines, a finding inconsistent with autism per se but also observed in PWS. Despite behavioral deficits, surprisingly, dendritic morphology and GAD67 are not altered in brains from fetal and postnatal KOs (Hashimoto et al., 2005; Hill et al., 2005). In contrast, double BDNF +/x SERT -/mutants display exacerbated anxiety in the elevated plus maze, greater elevation in plasma ACTH after stressful stimulus, and reduction in the size of dendrites of hippocampal and hypothalamic neurons in comparison to WT, SERT+/+ x BDNF+/and SERT-/-x BDNF +/+ mice (Ren-Patterson et al., 2005). Because autism is often considered to be a multigenic disorder, investigation of gene/gene interaction is a logical approach.

Urokinase Plasminogen Activator Receptor Knockout Mice

Variations in the MET gene encoding a receptor tyrosine kinase are associated with autism (Campbell et al., 2006). Moreover, post mortem analysis of cortical tissue from autistic individuals reveals decreased levels of MET protein in comparison to matched controls (Campbell et al., 2007). Disruption in signaling mediated by MET and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), may be particularly relevant to the etiology of the disorder because, in addition to playing a key role in the CNS during development and adulthood, it is also involved in gastrointestinal repair and regulation of the immune system, two other systems that are altered in autism (Vargas et al., 2005). The PI3K/Akt pathway is one of the prominent signaling cascades activated by MET, which thereby antagonizes PTEN function. In the CNS, HGF/SF-MET signaling promotes the migration of cortical interneurons during development (Powell, Mars, &

Levitt, 2001), contributes to cerebellar development and function (Leraci, Forni, & Ponzetto, 2002), stimulates dendritic growth in cortical neurons (Gutierrez et al., 2004), and induces protein clustering at excitatory synapses (Tyndall & Walikonis, 2006). Although genetic deletion of MET causes embryonic lethality, the KO of urokinase plasminogen activator receptor (uPAR), which exhibits reduced uPA activity (the protease required for the activation of HGF), is viable and displays a diminution in HGF levels and, as observed in autism, in MET levels. The uPAR KO mice display increased anxiety and are prone to seizures (Powell et al., 2003), features that are relevant to autism (Tuchman & Rapin, 2002). Whether these mice display deficits in any core symptoms of the disorder has not been reported. Gastrointestinal and immune pathology also needs to be assessed in these mutants.

Disrupted in Schizophrenia-1Variants

A balanced translocation between chromosome 1 and 11 t(1;11) (q42.2;q14.1) cosegregates with schizophrenia and related disorders in a large Scottish family (St Clair et al., 1990; Blackwood et al., 2001). Disrupted in Schizophrenia-1 (DISC1) is altered by this translocation (Muir et al., 1995; Millar et al., 2000; Millar et al., 2001), and there is an association between variations within the DISC locus and autism and Asperger Syndrome (Kilpinen et al., 2008). DISC1 is a scaffold protein, which, through interactions with various proteins (e.g., PDE4B, LIS1, NDEL1, NDE1, CIT, MAP1A), regulates cAMP signaling, cortical neuron migration, neurite outgrowth, glutamatergic neurotransmission, and synaptogenesis (Muir, Pickard, & Blackwood, 2008). There are a number of Disc1 mouse variants currently available: mice carrying a truncated version of

the endogenous Disc1 ortholog, transgenic lines with inducible expression of mutant human DISC1 (hDISC1), and lines carrying N-ethyl-N-nitrosourea-induced mutations in Disc1 (Chubb et al., 2008). Hippocampal neurons in mice with mutations of endogenous Disc1 display dendritic misorientation and reduced number of spines, as observed in the Fmr1 KO mice. These mice display a working memory deficit but do not show deficits in PPI or latent inhibition (Koike et al., 2006; Kvajo et al., 2008). Transgenic mice expressing hDISC1 in forebrain regions show a mild enlargement of the lateral ventricles in comparison to WT animals, and neurite outgrowth is decreased in primary cortical neurons from these mutants. These neuropathologies are associated with altered social interaction and enhanced spontaneous locomotor activity in male hDISC1 mice and with mild impairment in spatial memory in females (Pletnikov et al., 2008). Tests assessing repetitive behavior and ultrasonic vocalizations remain to be reported. Further study of neuropathology in the various strains will also be important.

Oxytocin and Vasopressin

Neuropeptides and their associated receptors play a central role in the regulation of complex social behaviors. Several lines of evidence suggest that functional alterations in these systems may contribute not only to social deficits in autism but also to repetitive behaviors. (1) A reduction in oxytocin (OT) plasma levels, associated with an elevation in the prohormone form, is observed in autistic children (Modahl et al., 1998; Green et al., 2001). Mixed results have been reported for OT plasma levels in high-functioning adult autistic patients(Jansen et al., 2006; Andari et al., 2010). (2) Intranasal infusion of OT reduces stereotyped behavior and improves eye contact, social memory and use of

social information in high functioning autistic patients (Hollander et al., 2003, 2007; Guastella et al., 2009; Andari et al., 2010). (3) Genetic variations in OT receptor and vasopressin receptor V1aR can be associated with autism (Donaldson & Young, 2008; Israel et al., 2008; Gregory et al., 2009). (4) Oxytocin receptor mRNA is decreased in post-mortem autism temporal cortex (Gregeory et al., 2009).

Current knowledge derived from studies in OT and OT receptor (OTR) KO mice underscore the subtle role of this system in aggression and anxiety. Thus, OT KO adult male progeny from homozygous crosses display high levels of aggression, whereas levels of aggression in OT KO adult male progeny from heterozygous crosses are either less pronounced or similar to WT mice, suggesting that absence of OT during prenatal stages modulates the development of aggression in adulthood (Ferguson et al., 2000; Winslow et al., 2000; Takayanagi et al., 2005). OT KO female mice also display exaggerated aggression under controlled stress conditions designed to mimic the natural environment, indicating a possible interaction between the postnatal environment and the OT system (Ragnauth et al., 2005). Similarly, OTRr KO adult males display elevated aggressive behavior, as well as deficits in social discrimination (Takayanagi et al., 2005). OT KO adult mice nevertheless display reduced anxiety in the plus maze and acoustic startle reflex, a finding inconsistent with autism. In addition, as infants, both OT and OTR KO males emit fewer USVs in the isolation test than WT animals, which is also suggestive of decreased anxiety during maternal separation, but is also consistent with the lack of communication in ASD. The OT KO mice fail to recognize familiar conspecifics upon repeated social encounters, although olfactory and nonsocial memory are intact (Winslow & Insel, 2002). This has been interpreted as an autism-like social deficit, although social

amnesia has not been described in autism. Comprehensive neuropathology remains to be reported in these strains. Given the implications for ASD in the human findings, further study of OT mutant mice is warranted, although striking species differences are apparent for OT and vasopressin, and their receptors (Insel, 2010).

Male V1aR KO mice exhibit deficits in olfactory social recognition and social interaction (Bielsky et al., 2004; Egashira et al., 2007). Similarly to OT and OTR KO mice, V1aR KO mice show reduced anxiety-like behavior in the elevated plus maze and the open field and the light/dark box, although high levels of anxiety are observed in the WT animals in comparison to other reports. No deficits in learning and memory in the Morris water maze or in PPI are detected, indicating that the face validity of this model is partial. V1bR KO adult females emit fewer USVs in a resident-intruder test. Although the number of USVs emitted by infant mutants is not affected during the conventional pup separation test, mutant pups fail to display maternal potentiation of USVs, which could suggest either a defect in a cognitive component or reduced anxiety (Scattoni et al., 2008). Although reduced anxiety is inconsistent with autism, V1bR antagonists could be tested to lower anxiety.

BTBR Mice

The BTBR mouse strain exhibits low levels of sociability at juvenile and adult ages, as well as abnormal social learning in the transmission of food preference test. Moreover, BTBR mice show a high level of spontaneous repetitive grooming, poor shift performance in a hole-board task, and a deficit in the water maze reversal task, which can be interpreted as the resistance to change in routine that is observed in autism (Bolivar,

Walters, & Phoenix, 2007; Moy et al., 2007; Yang, Zhodzishsky, & Crawley, 2007; McFarlane et al., 2008; Moy et al., 2008). Finally, BTBR pups separated from their mother emit more and longer USVs in comparison to C57 pups (Scattoni et al., 2008). Their repertoire of vocalizations is also narrower in comparison to pups from standard mouse strains. The latter observation is significant, as human infants later diagnosed with autism make unusual vocalizations (Johnson, 2008). However, one might expect to see lower rates of USVs in pups if modeling the ASD communication deficit. Such a deficit is reported in adult BRBR mice (Wohr et al., 2010). Detailed study of the fine structure of USVs, as well as their behavioral functions in adult mice, is an important area for future studies of animal models of psychiatric disease.

A recent study indicates that BTBR mice display an exaggerated response to stress that is associated with high blood levels of corticosterone in comparison to C57 mice (Benno et al., 2009). Thus, it is not clear whether enhanced stress causes or aggravates the behavioral phenotype of these mice. Key anatomical features of BTBR mice are the absence of the corpus callosum and a reduced hippocampal commissure. These deficits correlate with impaired contextual fear memory, which could arise from an increased susceptibility to de-potentiation. Otherwise, electrophysiological properties of hippocampal slices from BTBR (LTP, paired pulse facilitation, and basal synaptic transmission) are similar to those of C57 (MacPherson et al., 2008). Thus, several BTBR behaviors are consistent with autism, and the most striking anatomical feature in this strain is consistent with many, but not all, studies of the corpus callosum in autism (Amaral, Schumann, & Nordahl, 2008).

A difficulty with this line is that comparisons are necessarily made to other, unrelated mouse lines, and it is not clear to which line(s) BTBR should be compared. For instance, similar to BTBR mice, but unlike C57 mice, BALB/c mice display low social behavior, reduced USVs and reduced empathy-like behavior (Silverman et al., 2010). Because it is likely that there is a wide variety of genetic differences between such strains, comparing their behaviors is not equivalent to comparing behaviors and neuropathology between mutant and WT mice of the same genetic background. Nonetheless, the search for the genes causing behavioral phenotypes is ongoing, and a single nucleotide polymorphism in Kmo, which encodes kynurenine 3-hydroxylase, has been found in BTBR mice when compared to unrelated strains (McFarlane et al., 2008). This enzyme regulates the synthesis of kynurenic acid, a neuroprotective compound for which levels are abnormal in other neuropsychiatric diseases, including schizophrenia.

Myocyte Enhancer Factor 2 KO mice

A recent homozygosity mapping study of autism loci identified several candidate genes that are regulated by myocyte enhancer factor 2 (MEF2) transcription factors (Morrow et al., 2008). MEF2 factors mediate synapse elimination. Conditional KO of Mef2c at the neural stem cell stage yields mice with fewer, smaller, and more compacted neurons that exhibit what is interpreted as immature electrophysiological network properties. It is of interest that these mice display marked paw-clasping stereotypy, possibly altered spatial memory, and complex changes in anxiety tests when tested as adults (Li et al., 2008). Indeed, such behaviors resemble those observed in Mecp2 mutants. In addition, a recent study revealed that active FRMP is required for MEF2-

dependant synapse elimination, thus further pinpointing the molecular factors at play in regulating synapse number (Pfeiffer et al. 2010).

Copy Number Variations

Copy number variations (CNVs) are DNA fragments whose number is altered by deletion or duplication between various individuals. They are thought to account for a significant proportion of normal phenotypic variation within the human population (Freeman et al., 2006). Several recent studies have indicated that de novo CNVs associate with autism. Interestingly, some of the genes identified within the loci subject to CNVs in autism are related to synaptic or neuronal activity (e.g., SHANK3, NLGN4, and NRXN1) (for reviews, see Abrahams & Geschwind, 2008a; Cook & Scherer, 2008). Thus, genome-wide investigation of CNVs might further implicate or reveal novel candidate genes in autism, for which rodent mutant lines can be developed.

Lesions

Autism is a common occurrence in children with brain lesions caused by hemorrhage or tumor (Asano et al., 2001; Limperopoulos et al., 2007). One of the examples is TSC, a genetic disorder that causes benign lesions or tumors to form in many different organs, including the brain. MRI and positron emission tomography (PET) demonstrate correlations between abnormalities in the cortex and communication deficits, whereas changes in subcortical circuits correlate with stereotypies and lack of social interaction (Asano et al., 2001). Therefore, the study of autism in children with TSC and the development of animal lesion models may provide clues about autistic behavior.

Although brain lesions have commonly been used in animal models to study the circuitry underlying behaviors (Lavond & Steinmetz, 2003), interpretation of such results can be complex. First, the loss of a behavior does not prove that the lesioned brain area is the originating source for brain activity associated with that behavior. Second, lesions often damage axons passing through the brain area of interest, giving rise to erroneous conclusions about that brain region's importance in the behavior. Third, behavioral testing is necessarily performed on subjects responding to injury with various inflammatory and regenerative mechanisms whose influence on behavior is poorly understood. More recent advances in the ability to silence particular neuronal populations using genetic techniques promise much greater sophistication in unraveling the circuitry underlying behavior.

Amygdala

The amygdala has reciprocal connections with many areas, including the orbital and medial prefrontal cortex and the hippocampus, which are implicated in autism. Several post mortem studies have demonstrated amygdala abnormalities in autistic subjects, such as altered developmental trajectory and fewer neurons (Amaral et al., 2008). In case reports, children with severe temporal lobe damage resulting from viral encephalitis, tumors, or other factors developed autistic symptoms (Sweeten at al., 2002). In children with TSC, symptoms of autism are strongly related to the presence of tubers in the temporal lobe (Gillberg et al., 1994). These findings, along with recent functional neuroimaging data, led Baron-Cohen to develop an "amygdala theory of autism" (2000), which suggests a crucial role for the amygdala in the impairment of social behavior. In

addition, patients with amygdala lesions (Adolphs, 2003) and individuals with autism (Adolphs et al., 2001) appear to have similar deficits in recognizing complex emotions in facial expressions. Neonatal ibotenic acid lesion of the amygdala in the rat has been proposed as an animal model of neurodevelopmental disorders such as autism (Daenen et al., 2001; 2002a; 2002b; Diergaarde et al., 2004). In this model, animals display increased latency to play with social partners decreases duration of contact and hyperactivity as well as decreases adaptation and habituation to the open field, which is interpreted as locomotor stereotypy and low anxiety (Daenen et al., 2001; 2002a). Thus, excitotoxic lesions of the amygdala in the neonatal rat produce multiple behavioral abnormalities relevant to autism. This conclusion differs from that found in similar nonhuman primate studies (Amaral et al., 2003). Moreover, humans with amygdala lesions do not display deficits in social interaction like those seen in autism (Amaral et al., 2003).

Hippocampus

Limbic system dysfunction is fundamental to autism (Amaral et al., 2008). Neonatal ibotenic acid lesion of the hippocampus in the rat causes locomotor stereotypy in the open field test, but there are mixed results concerning deficits in social behavior early in life or in adulthood (Daenen et al., 2002b; Silva-Gomez et al., 2003) In rodent models, hippocampal lesions grossly impair memory, and autistic subjects display a selective deficit in hippocampal-dependent memory (Lathe, 2006). Because there are only a few studies analyzing social behavior in hippocampus-lesioned rats, and the

damage in this area has no or only a temporary effect in monkeys (Amaral et al., 2003), more work is required in this area.

Prefrontal Cortex

Patients who suffer damage to the prefrontal cortex have problems in making decisions and in behaving appropriately in social situations where empathy and social and moral reasoning are appropriate (Anderson et al., 1999; Koenigs et al., 2007). There is also evidence that both the medial temporal lobe and dorsolateral prefrontal cortex are implicated in autism (Amaral et al., 2008). Moreover, lesions to the orbital and medial prefrontal cortex in nonhuman primates cause abnormal social personality expression, decision making, and loss of position within the social group (Butter & Snyder, 1972). Lesioning prefrontal cortex also provokes a decrease in positive social behaviors (grooming, huddling, and near-body contact) and socially communicative facial, vocal, and postural behaviors, as well as an increase in inappropriate social interactions (Myers et al., 1973). Consistent with these results are ibotenic acid lesion studies of the medial prefrontal cortex in rats (Schneider & Koch, 2005). In addition, the lesion-induced disturbances of juvenile play behavior in rodents may also contribute to the social deficits observed in adult animals, because play-fighting is important for the development of communicative skills and appropriate behavioral patterns. Further work on the rodent model showed that neonatal lesion of the medial prefrontal cortex leads to reduced anxiety in the elevated plus maze, increased motor activity in open field, and, more interestingly, perseverative behavior in a reward-related test of operant behavior

(Schwabe et al., 2006). This is in line with the behavioral changes reported for cerebellum lesion models.

Cerebellum

The cerebellum is involved not only in the regulation of motor skills but also in higher functions, including cognition, language, and emotional expression (Turner et al., 2007). Adults and children with cerebellar lesions have high social isolation, communicative disturbance, and deficits in cognitive processing (Eluvathingal et al., 2006; Limperopoulos et al., 2007). A common finding in autism is a loss of PCs (72% of cases; Palmen et al., 2004; 79% of cases; Amaral et al., 2008). Therefore, surgical or toxin lesions of the cerebellum are of particular interest in studying animal models of autism. In addition to cognitive dysfunction reported in a rat model of cerebellum lesion (Gaytan-Tocaven & Olvera-Cortes, 2004), there are two rat models displaying perseverative behavior, which is common in autism and is expressed as insistence on sameness as well as inability to understand and cope with novel situations. This kind of behavior is found in a rat model of early (P10) midline cerebellar lesion (Bobee et al., 2000). In addition, these animals display elevated spontaneous motor activity and exhibit lack of attention to environmental distractors. Unlike the autism phenotype, however, the lesioned animals are neophilic and less anxious than controls. Vermis-lesioned rats show a decreased coefficient of learning during extended training of an instrumental task, which is interpreted as perseverative behavior (Callu et al., 2007).

In sum, the lesion models support dysfunction in autism in several areas: the prefrontal cortex, the temporal lobe and the cerebellum. This may be because an early

defect in the functioning of these areas of the brain alters the development of multiple other areas, however. Behavioral analysis has focused primarily on motor tasks and anxiety. These lesion models display hyperactivity and low anxiety in the open field, which is unlike the autism phenotype (Lathe, 2006). No changes, or decreased general social activity, are reported for rodent models with lesions in the amygdala, prefrontal cortex, or hippocampus. Early damage to the amygdala or the prefrontal cortex results in altered juvenile play behavior, the earliest form of non-mother-directed social behavior in rodents. Thus far, these lesion models display two types of behaviors impaired in autism—namely, a deficit in social behavior and stereotypy. It will be important to assay other types of social and communicative behavior (for example, ultrasonic vocalizations in different social contexts), as well as to compare the changes in brain pathology, biochemistry, and gene expression to those seen in autism.

Conclusions

Given that the background genotypes of the rat and mouse strains that are used for experiments are somewhat arbitrary, and that genotype is very important in autism, it is remarkable that both behaviors and neuropathology consistent with autism can be reproduced in rodents. This issue of background genotype could potentially become increasingly important as more sophisticated models incorporating human genetic variants are introduced. Although no single rodent model has yet been thoroughly studied at all levels of investigation, several models have already been explored in some depth. Among those that display face and construct validity are the maternal valproate and maternal infection models of environmental risk factors, as well as the NLGN-4, Frmr1,

CNTNAP2, and MeCP2 models of genetic risk factors. Nonetheless, no model has been thoroughly investigated with all available experimental tools, including behavior, histology, biochemistry, electrophysiology, and imaging.

Challenges and future directions

The fine structure of ultrasonic vocalizations, as well as their behavioral functions in adult mice, are intriguing areas for future study of potential communication abnormalities. Theory of mind, the ability to intuit another's thinking, is an important deficit in autism. An analogous test of empathy can be done in mice, where observing cage mates experience stressful experiences can alter the witness' responses to later tests (Langford et al., 2006; Chen, Panksepp & Lahvis, 2009). This assay should be widely tested in the available environmental and genetic models. To reach their full potential in mimicking the human situation, models of candidate genes should carry the variant identified in the human studies, not just a KO of the gene.

It is now possible to test the hypothesis that the full autism phenotype may emerge from environmental risk factors acting on susceptibility genotypes. Multi-electrode recording, functional imaging, and immediate early gene activation studies will be important for mapping patterns of functional activity in rodent models and comparing it to fMRI data from human subjects. Translational, preclinical studies in several mouse models have already stimulated small clinical trials in ASD-related disorders. Although this will likely accelerate in the near future, significant species differences will undoubtedly lead to failures along the way.

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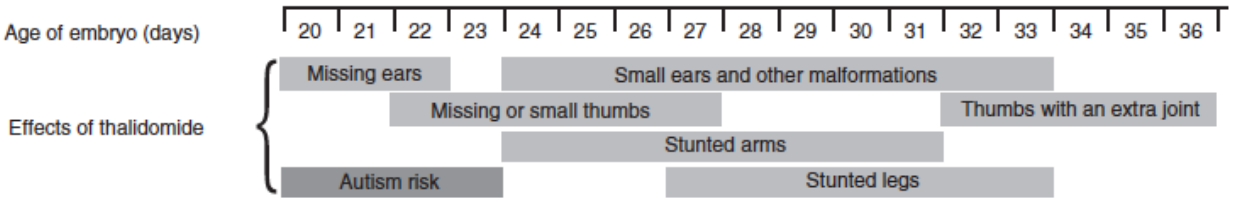

Figure 52–1. Timeline of Birth Defects Caused by Thalidomide, and the Critical Period of Vulnerability to Autism. The specific dysmorphologies in the offspring depend on the precise timing of drug ingestion. The fact that autistic features were only seen in offspring of a particular set of dysmorphologies indicates that the period of thalidomide-induced autistic features corresponds to days 20-23 of human gestation. Graphic after Rodier, PM. (2000) The Early Origins of Autism. Scientific American , 282 (2), 56–63

Table 52–1.
Behavioral Assays Used in Rodent Models of Autistic Features

Behavioral Paradigm	Testing Environment	Dependent Variables
SOCIAL BEHAVIOR		
sociability	three-chambered cage with inanimate object versus social object (mouse)	time spent in each chamber; number of approaches
preference for social novelty	three-chambered cage with familiar mouse versus unfamiliar mouse	time spent in each chamber; number of approaches
ultrasonic vocalizations	empty cage; pups are briefly isolated from mothers	number of calls, frequency of calls, type of call
LEARNING AND MEMORY		
Cincinnati water maze (multiple T maze)	maze design consisting of nine interlinked T-units submerged in water; mice are trained to swim to the escape platform	latency to find platform; time spent swimming in the trained quadrant of the pool as compared to time spent in the other quadrants
Morris water maze	circular pool of opaque water; mice are initially trained to swim to visible platform and then to submerged hidden platform	latency to find platform; time spent swimming in the trained quadrant as compared to time spent in the other three quadrants
eyeblink conditioning	chamber with tone generator for administration of a series of tones (conditioned stimulus, CS) followed by a periorbital shock (unconditioned stimulus)	occurrence of eyeblink as measured by EMG eyeblink signal (conditioned response, CR); amplitude of CR; latency between CS and onset of CR; latency between CS and peak of CR
novel object exploration	brightly lit open arena; in first trial, mice are exposed to two objects; in the second trial, one of the two objects is exchanged for a novel object	investigation of novel object; time spent with the novel object
novel location exploration	brightly lit open arena; in the first trial, mice are exposed to two objects; in the second trial, one of the two objects is moved to a new location	investigation of moved object; time spent with moved object
latent inhibition	chamber with tone generator for administration of a series of tones followed by an adversive stimulus (mild foot shock)	whole body flinch amplitude; freezing in context environment and after tonal cues
SENSORIMOTOR GATING		
acoustic prepulse inhibition	cage with tone generator for administration of serious of prepulse tones followed by startle stimulus	whole body flinch amplitude; freezing
ANXIETY/LOCOMOTION		
open field exploration	brightly lit open arena	center duration, number of center entries, total distance traveled, horizontal activity, vertical activity (rearing)
amphetamine-induced locomotion	brightly lit open arena	total distance traveled, horizontal activity, vertical activity (rearing)

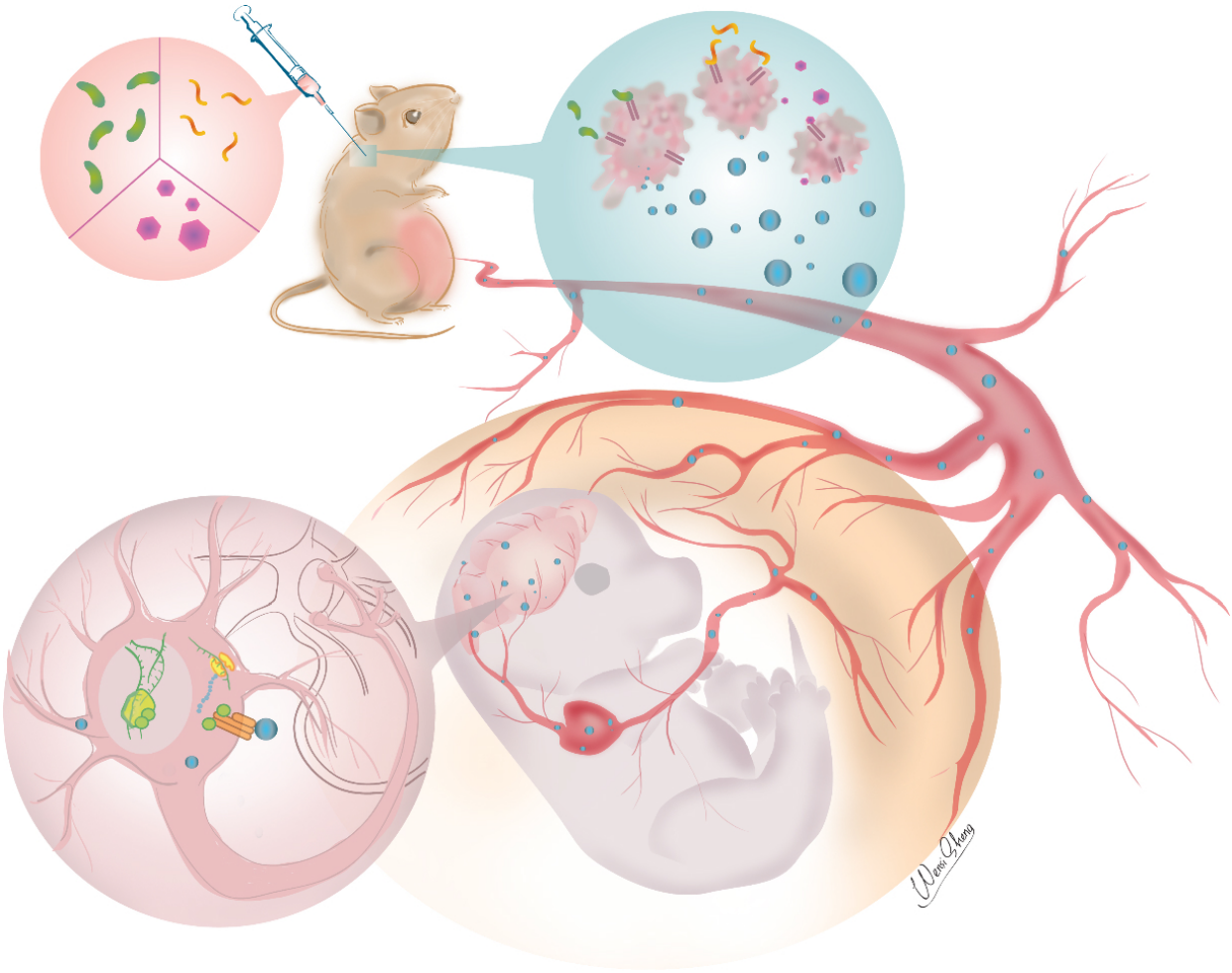

Figure 52–2. Cytokines Produced by Activation of the Maternal Immune System Can Alter Fetal Brain Development. Various types of infection (bacterial, viral, parasitic) in pregnant rats or mice can be mimicked by injection of LPS or poly(I:C). These activate cells to produce cytokines, which travel in the blood to the placenta, where they can activate cells. The cytokines can also cross the placenta into the fetal circulatory system and activate cells in the fetal brain.

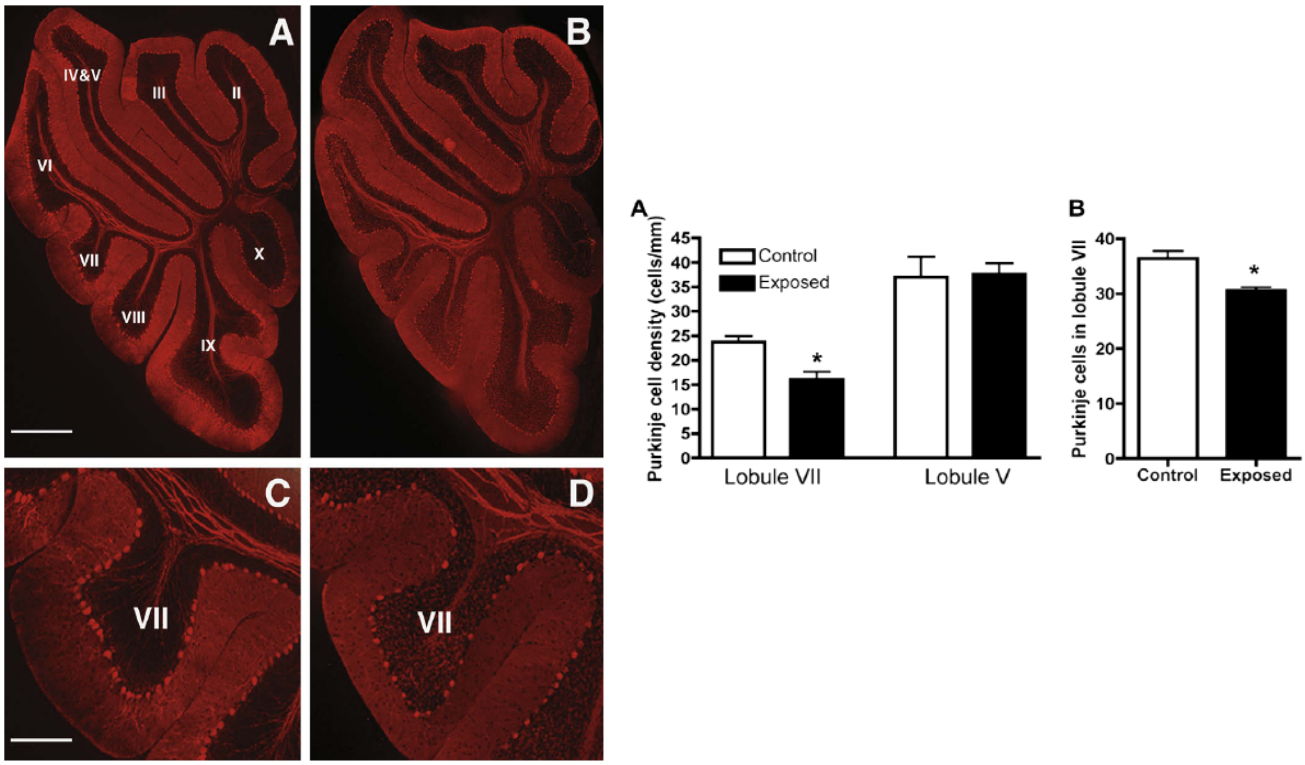

Figure 52-3. Maternal infection causes a spatially-restricted Purkinje cell deficit. Adult offspring of mice given a respiratory infection with influenza virus at midgestation display a deficit in Purkinje cells in lobules VII but not in other lobules. Left: Calbindin staining of adult cerebella from offspring of control (A,C) and infected mothers (B,D) reveals a deficit in lobule VII of the latter. Panels C and D (bar = 200 mm) are higher magnification views of panels A and B (bar – 800 mm). Right: (A) Quantification of the linear density of Purkinje cells reveals a 33% deficit in lobule VII of the adult offspring of infected mothers, while no difference from controls is found in lobule V. (B) A similar, localized deficit is observed in postnatal day 11 offspring of infected mothers. (Reprinted from Shi et al., 2009, with permission)

Mouse Mutant Relevant to Autism	Rescuer Mouse Mutant	Phenotype of the Double Mutant		
		Behavior	Neuropathology	Additional Parameters
FMR1 knockout (KO) mice	**Dominant negative PAK transgenic mice** 40% inhibition of the catalytic activity of PAK (Hayashi et al., 2007)	Hyperactivity, stereotypy, and hypoanxiety in open field are rescued	Spine density partially restored	
		Memory deficits restored	Spine length comparable to WT	
			Reduced cortical LTP is rescued	
	***Grm5* mutant mice** 50% reduction in mGluR5 expression (Dolen et al., 2007)	Exaggerated inhibitory avoidance extinction rescued	Spine density comparable to WT	Ocular dominance plasticity rescued
			Increased protein synthesis in the hippocampus is prevented	Growth increase at P30 rescued
			Audiogenic seizures attenuated	Macroorchidism not rescued
Germline *Mecp2* mutant	**Conditional BDNF-over-expressing transgenic mice** Increase in BDNF expression (Chang et al., 2006)	Improvement in the running wheel assay (locomotor function)	Neuronal activity indistinguishable from WT	Extension of the lifespan
			Modest increase in brain weight	
Angelman syndrome mutant	**CaMKII-T305V/T306A mutant mice** Mutations in CaMKII that prevent its auto-phosphorylation (Van Woerden et al., 2007)	Rotarod performance indistinguishable from WT	Absence of audiogenic seizures	Increase in body weight rescued
			75% reduction of propensity for seizures	
		Context-dependant memory restored (hippocampal learning deficit rescued)	Long-term potentiation rescued	
		Improved water maze performance		

Table 52-2. Amelioration or rescue of some features of the autism-like phenotype in double mouse mutants. Phenotypic improvements or reversals indicate functional (direct or indirect) interactions between the targeted genes, while persistence of one phenotype suggests that additional genes contribute to the full phenotype. Investigation of genetic interactions provides insight into the molecular pathways underlying particular facets of autism, and may suggest novel therapeutic targets.

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Chapter 3

Activation of the maternal immune system as a risk factor for neuropsychiatric disorders

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Abstract

Maternal infection can alter the intrauterine environment, placing the developing fetus at risk. Serious infections of the mother or the fetus can cause severe problems or even miscarriage. The more common and seemingly benign infections, such as influenza, are not associated with such severe outcomes. However, recent work has shown that maternal inflammation associated with any infection has the potential to alter fetal brain development. Epidemiological studies have demonstrated a strong association between maternal infection and subsequent development of mental illness in the offspring. Mouse models of maternal infection have shown long-lasting changes in the brains of offspring following maternal immune activation, as well as behavioral abnormalities related to human mental illness. Recent work has begun to elucidate the mechanisms through which the activated maternal immune system alters fetal brain development. We review the evidence from human and rodent studies showing that maternal infection is a risk factor for mental illness, and describe the initial steps in the molecular mechanism mediating the effects of maternal immune activation on fetal brain development.

Introduction

Surprisingly little is known about the subtle changes in the brain that result in mental disorders such as schizophrenia and autism. These disorders are defined by behavioral symptoms. A child presenting before the age of three with abnormal language acquisition and reciprocal social interactions, as well as repetitive, stereotyped behaviors is diagnosed as autistic. A young adult who reports positive (hallucinations or delusions) and negative (depression, apathy, social withdrawal) symptoms is diagnosed as schizophrenic. Although these symptoms are dramatic, the brain looks grossly normal, and even upon microscopic examination the neuropathology is subtle. However, studies of post-mortem protein and gene expression have identified many changes, including molecules involved in immune regulation, synaptic function and myelin. Unfortunately, our understanding of the normal function of the brain is not sufficiently developed to form a coherent model of the malfunction in these disorders.

Another approach to these disorders is to identify and study the risk factors that contribute to their development. Despite the postnatal onset of the clinical symptoms, evidence suggests that schizophrenia and autism have their origins in early brain development. Retrospective examination of school records and home videos of children who subsequently display these disorders portray their early behaviors as "pre-schizophrenic" or "pre-autistic" (Palomo, Belinchon, & Ozonoff, 2006). Moreover, postmortem examination of the brains of at least some affected individuals reveals subtle changes in the number, distribution or alignment of neurons, suggesting abnormalities in development *in utero* (Lewis & Levitt, 2002). Therefore, taking a bottom-up approach of

identifying risk factors and tracing their mechanism of action may be a productive path towards understanding these complex disorders with heterogeneous symptoms.

Risk factors contributing to schizophrenia and autism

While genetics clearly plays a major role in schizophrenia and autism, the complex nature of inheritance continues to puzzle researchers. Autism is often cited as one of the most heritable mental diseases, with twin studies often cited as evidence: while ~90% of identical twins are concordant for autism, that number falls to about 10% for fraternal twins. Similar data exists for schizophrenia, with concordance rates of ~50% in monozygotic versus ~17% in dizygotic twins (Lewis & Lieberman, 2000). Despite the relatively high heritability, however, only about 5-10% of autism cases can be can be attributed to known chromosomal abnormalities or single gene mutations. Similarly, with notable exceptions (e.g., DISC1), very few cases of schizophrenia can be traced to a known genetic cause. Moreover, large, statistically powerful genetic association studies of patient populations have failed to identify more than a handful of implicated genetic variants, and these carry a small effect size (Grice & Buxbaum, 2006; Sykes & Lamb, 2007).

Several theories, reviewed elsewhere, attempt to reconcile the high apparent heritability of mental illness with the difficulty of identifying genetic risk factors. An early explanation is the "multiple interacting genes" hypothesis, which states that the inheritance involves multiple genes, each of which displays incomplete penetrance (see, for example, (Grice & Buxbaum, 2006)). A more recent theory posits that *de novo* genetic mutations and chromosomal rearrangements contribute to risk. Evidence

showing an elevated rate of microdeletions and microduplications in autistic and schizophrenic patient samples, as well as associations of autism with increasing parental age lends credence to this hypothesis (Cook & Scherer, 2008). While *de novo* microdeletions and duplications would explain why so few candidate single gene mutations have been identified in studies of multiple populations, they do not explain the high heritability estimates of these disorders. Similarly, epigenetic regulation is postulated to be involved in autism and schizophrenia (for review see (Schanen, 2006)).

Environmental factors may contribute to the difficulties in genetic analyses. Proof of principle for a role of environmental factors is provided by studies showing that exposure to thalidomide, valproic acid or prenatal infections strongly increase the risk for autism and schizophrenia in the offspring. Therefore, it is possible that environmental risk factors contribute to genetic susceptibility or that environmental factors require a susceptibility genotype to increase risk. It will be important to understand all of the risk factors, genetic, epigenetic and environmental, in order to develop a complete understanding of, and more effective treatments for, these disorders.

Maternal infection: an environmental risk factor

Maternal infection is among the most studied and best-established non-genetic risk factor for schizophrenia. The connection between schizophrenia and maternal influenza infection was first indicated by the work of Mednick *et al.* (Mednick, Machon, Huttunen, & Bonett, 1988). Since then, over 25 studies have examined the rate of schizophrenia in populations which were *in utero* during influenza epidemics, and the majority found an increased incidence among the exposed offspring (reviewed by (Bagalkote, Pang, & Jones,

2001)). However, such ecological studies are population-based; they are unable to document a one-to-one relationship between respiratory infection in individual mothers and later development of schizophrenia in their children. To overcome this limitation, Brown, Susser and colleagues examined a large pool of archived maternal serum samples that were collected in the 1960s and linked to detailed medical records of both mothers and children. They found that in cases where they were able to confirm maternal influenza infection by assaying the serum, the offspring were 3-7 times more likely than controls to develop schizophrenia (Brown, Begg, et al., 2004). Due to the high prevalence of influenza, they estimate that up to 21% of all schizophrenia cases may be traced to maternal influenza infection. A separate study examining Danish medical records found an 8-fold risk increase in schizophrenia risk with maternal influenza infection (Byrne, Agerbo, Bennedsen, Eaton, & Mortensen, 2007). Further studies have found links between elevated levels of cord blood cytokines and development of schizophrenia, strengthening the association of influenza infection with schizophrenia risk (Brown, 2006; Brown, Hooton, et al., 2004). In addition, serological studies have found links between schizophrenia and maternal toxoplasmosis (Brown et al., 2005; Mortensen et al., 2007), and rubella (Buka et al., 2001), genital/reproductive (Babulas, Factor-Litvak, Goetz, Schaefer, & Brown, 2006), and bacterial infections (Sorensen, Mortensen, Reinisch, & Mednick, 2009). Thus, many different pathogens increase the risk of mental disease in the offspring.

An important insight into the heterogeneity of the schizophrenia phenotype and its risk factors was revealed by further study of the population being monitored by Brown *et al*. Comparison of schizophrenics born to infected versus non-infected mothers showed both anatomical (enlarged cavum septum pellucidum) and cognitive differences between the two

groups (Brown, in press; Brown et al., 2009). It will be interesting to see if these two groups also display divergent responses to anti-psychotic drugs, and what the distribution of candidate gene variants for schizophrenia may be.

Similar, but considerably less extensive, epidemiological data exist for autism. Rubella epidemics in the 1960s were associated with a striking elevation in the incidence of autism in children born to infected mothers (Chess, 1977; Desmond et al., 1967). Studies of other maternal infections, such as toxoplasmosis, syphilis, varicella and rubeola, also support the idea that maternal infection can be a risk factor for autism (for a review, see (Ciaranello & Ciaranello, 1995)), although the number of individuals in these studies is very small.

Animal models of immune activation

Maternal influenza infection

Both the epidemiologic data and the serologic studies show that infection during early-to-mid pregnancy results in the greatest schizophrenia risk in the offspring (Brown, Begg, et al., 2004; Brown & Susser, 2002). In order to produce an animal model that most closely replicates the human data, Shi *et al.* (Shi, Fatemi, Sidwell, & Patterson, 2003) inoculated mice intra-nasally with a human H1N1 influenza virus on day 9.5 of pregnancy, which is the rough equivalent of human early second trimester. The pregnant dams develop lung pathology, show noticeable sickness behavior, and have elevated serum levels of several immune markers for approximately seven days, covering the second half of the pregnancy. The adult offspring of influenza-infected mice appear superficially normal, but

display several behavioral abnormalities that are highly relevant to schizophrenia and autism. For example, they have lower pre-pulse inhibition (PPI) than controls, and this deficit is rescued by acute treatment with antipsychotic drugs. PPI is abnormal in schizophrenia (Turetsky et al., 2007; Wynn et al., 2004) and autism (Perry, Minassian, Lopez, Maron, & Lincoln, 2006), as well as in other mental disorders such as depression. The offspring also display heightened anxiety in the open field, and they show a reduced number of social interactions when placed in an open field with an unfamiliar, same-sex conspecific (Shi, et al., 2003). These offspring also display several histological abnormalities that are reminiscent of those found in autism and/or schizophrenia, such as reduced reelin (Fatemi et al., 1999) and parvalbumin (Meyer, Nyffeler, Yee, Knuesel, & Feldon, 2008) immunoreactivity. They also display a spatially restricted deficit in Purkinje cells (PCs) in lobules VI and VII of the cerebellum (Shi, Smith, Malkova, Tse, & Patterson, 2008), which is consistent with the most commonly reported histological abnormality in autism (reviewed by (Amaral, Schumann, & Nordahl, 2008)).

Maternal Immune Activation (MIA)

The fact that a wide variety of pathogens are able to increase schizophrenia risk in the offspring suggests that these pathogens alter fetal development by a common mechanism. Direct infection of the fetus seems an obvious hypothesis. However, most of the implicated infections are confined to specific areas of the body, and do not involve the fetus. For example, influenza infection is confined to the lungs, since the membrane proteins that allow viral entry into a cell are found only in the lungs (Taubenberger & Morens, 2008). In the mouse model of maternal influenza infection, using a sensitive RT-

PCR assay that can detect as little as one plaque-forming unit of virus, no virus is detected in the exposed fetuses (Shi, Tu, & Patterson, 2005). Maternal immune activation is also a plausible mechanism; all of the implicated pathogens trigger a maternal immune response, which will alter the biochemical environment of the fetus. In strong support of this hypothesis, two rodent models have been developed in which behavioral deficits are induced in adult offspring by directly activating the maternal immune system in the absence of infectious organisms.

Maternal poly(I:C) administration

Poly(I:C) is a synthetic, double-stranded RNA that is a potent agonist of the toll-like receptor-3 (TLR-3). Double-stranded RNA is regarded by the innate immune system as a signal of viral infection; thus the activation of TLR-3 induces a pro-inflammatory, NF-*k*B-dependent cascade that results in the production of anti-viral cytokines and chemokines. Injection of poly(I:C) in the pregnant rodent at mid-gestation produces offspring that are remarkably similar to the offspring of mice given a flu infection. These offspring display deficits in PPI, latent inhibition, open field exploration and social interaction that are almost identical to those exhibited by influenza offspring (Smith, Li, Garbett, Mirnics, & Patterson, 2007). Moreover, investigators using the poly(I:C) model of MIA have shown that the PPI and latent inhibition deficits occur in post-pubertal but not in juvenile rats, mimicking the adult-onset nature of schizophrenia (Piontkewitz, Weiner, & Assaf, 2007; Zuckerman, Rehavi, Nachman, & Weiner, 2003). These deficits are reversed by acute anti-psychotic drug treatment. Maternal poly(I:C) also causes other changes relevant to autism and/or schizophrenia: a PC deficit [31], increased dopamine

release and turnover (Ozawa et al., 2006), reduced parvalbumin expression (Meyer, et al., 2008), and increased $GABA_A$ receptor expression (Nyffeler, Meyer, Yee, Feldon, & Knuesel, 2006) in the adult offspring.

Maternal LPS administration

MIA can also be induced by the injection of bacterial lipopolysaccharide (LPS), a natural ligand for TLR-4. Intrauterine bacterial infection is commonly associated with preterm birth, obstetrical complications and cerebral palsy (R. Romero, Gotsch, Pineles, & Kusanovic, 2007). There is evidence linking maternal bacterial infection with schizophrenia in the offspring (O'Callaghan et al., 1994; Watson, Kucala, Tilleskjor, & Jacobs, 1984), and ligands binding TLR-4 activate many of the same signaling pathways as TLR-3. The specific combination of cytokine and chemokine activation for TLR-4 is different than for TLR-3, but like poly(I:C), LPS produces a very strong, transient immune activation. Thus, bacterial and viral infections can induce similar responses. Indeed, some of the behavioral abnormalities seen in the offspring of poly(I:C)-treated mothers are also observed in the offspring of LPS-treated mothers. PPI deficits are often reported, as are increased anxiety, social interaction deficits, and abnormalities in dopamine-related behaviors (Borrell, Vela, Arevalo-Martin, Molina-Holgado, & Guaza, 2002; Fortier et al., 2004; H. M. Golan, Lev, Hallak, Sorokin, & Huleihel, 2005; Hava, Vered, Yael, Mordechai, & Mahoud, 2006; R. Romero, et al., 2007). Histological findings in the LPS model include fewer, more densely packed neurons in the hippocampus, increased microglial and GFAP staining, altered tyrosine hydroxylase staining, and decreased myelin basic protein staining (Borrell, et al., 2002; Cai, Pan,

Pang, Evans, & Rhodes, 2000; H. M. Golan, et al., 2005; Ling et al., 2004; Paintlia, Paintlia, Barbosa, Singh, & Singh, 2004).

Other protocols for maternal immune activation

Bacteria commonly associated with periodontal disease have been isolated from preterm placentas, and epidemiological evidence links schizophrenia risk to both periodontal disease and preterm birth (Bobetsis, Barros, & Offenbacher, 2006). Mouse models involving injection of periodontal bacteria into a pregnant dam result in fetal growth restriction and mortality (Bobetsis, et al., 2006). Injection of LPS directly into the fetus also results in low birth weight and fetal mortality (reviewed by (Wang, Rousset, Hagberg, & Mallard, 2006)).

There is also a large body of research on maternal stress causing abnormal behavior in the adult offspring. Epidemiological evidence links maternal stress caused by diverse stimuli such as war or death of a loved one to schizophrenia in the offspring (reviewed by (Beydoun & Saftlas, 2008)), and animal models document behavioral changes in the offspring of rats exposed to daily restraint stress. Barbazanges *et al.* (Barbazanges, Piazza, Le Moal, & Maccari, 1996) showed that this effect of stress is mediated by glucocorticoid, which can also have strong modulatory effects on the innate immune system.

Mechanisms underlying MIA-induced behavioral abnormalities

Cytokines are small (8-30 kD) signaling proteins that are released into the serum in response to a wide range of stimuli including infection, immune activation and stress.

Thus, even though an influenza infection is confined to the lungs, cytokines produced there will have access to the placenta and fetus. Cytokines signal through several key kinase pathways, including JAK/STAT, NF-*k*B, and MAPK. During brain development, these same pathways control functions such as neuronal differentiation and migration, axon pathfinding and synapse formation. Moreover, many cytokine receptors are expressed in both developing and mature neurons and can cause morphological and functional changes in those cells (Bauer, Kerr, & Patterson, 2007; Gilmore, Jarskog, Vadlamudi, & Lauder, 2004; Jankowsky & Patterson, 1999). If cytokines are able to cross from maternal circulation into the fetal brain, the resultant disruption in neuronal homeostasis could account for the long-term effects in the offspring.

Mounting evidence shows that the maternal inflammatory response does, in fact, cross the placenta and affect the fetus. Radiolabeling studies have demonstrated that labeled IL-6 and IL-2 enter the fetus when injected into pregnant dams (Dahlgren, Samuelsson, Jansson, & Holmang, 2006; Ponzio, Servatius, Beck, Marzouk, & Kreider, 2007). Several groups have shown increased cytokine protein in the fetal brain following MIA induced by LPS or poly(I:C). Increases in cytokine mRNA have been found in fetal brain as well, making it unclear if the cytokines found in the fetal brain are of maternal or fetal origin. Both protein and mRNA of IL-1β, IL-6, tumor necrosis factor α (TNF-α) and IL-10 are upregulated at various time points ranging from 2-24 hours post-injection (see Table I). This response seems to be dose-dependent, as groups that use more severe treatments (i.e., higher doses of poly(I:C) or LPS; intravenous versus intraperitoneal injection) tend to see more robust increases in fetal brain cytokines. Recent work from our group has found evidence of induction of several response genes downstream of IL-6,

confirming that cytokines in the fetal brain are having significant biological effects (E. Hsiao and P.H. Patterson, unpublished).

Manipulation of cytokine expression during MIA has demonstrated that cytokine signaling plays a key role in the development of abnormal behavior. Meyer et al. were able to prevent the development of behavioral abnormalities in the offspring of MIA dams by overexpression of IL-10, an anti-inflammatory cytokine (Meyer et al., 2007). IL-10 can also protect against white matter damage caused by maternal *E. coli* infection (Pang, Rodts-Palenik, Cai, Bennett, & Rhodes, 2005). Similarly, anti-TNFα antibodies can reduce the growth restriction and fetal loss caused by LPS injection, and conversely, injection of TNFα can mimic the effects of LPS (Silver, Lohner, Daynes, Mitchell, & Branch, 1994; Xu et al., 2006).

Work from our laboratory has demonstrated that IL-6 plays a critical role in the effect of poly(I:C)-induced MIA (Smith, et al., 2007). We screened for cytokines that might mediate the effect of MIA by co-injecting poly(I:C) and various anti-cytokine antibodies into pregnant mice. We found that co-injection of anti-IL-6 prevented the behavioral deficits caused by poly(I:C). Antibodies to IL-1β, TNFα, and interferon-γ had no significant effect. Moreover, blocking IL-6 also prevented maternal poly(I:C)-induced gene expression changes in the adult offspring brain, as measured by RNA microarray. In addition, IL-6 knockout mice did not show behavioral deficits after MIA. Conversely, a single injection of recombinant IL-6 (but not the other cytokines tested) into pregnant mice caused behavioral deficits similar to MIA. Samuelsson et al. have reported long term effects of multiple IL-6 injections in pregnant rats (Samuelsson, Jennische, Hansson, & Holmang, 2006); the offspring showed deficits in learning as well as elevated serum

levels of IL-6 as adults, which may mimic the ongoing immune activation observed in mental illness (see below).

These animal models demonstrate that MIA, particularly maternal IL-6 activation, causes long term changes in the behavior of the offspring that are reminiscent of symptoms seen in human mental illnesss. But how could a transient increase in a single immune signaling molecule such as IL-6 (albeit at a critical time in development) precipitate such life-long changes? It is possible that the development of a homeostatic regulatory mechanism is disrupted, leading to dysregulation of gene expression not only in the brain, but in the immune system as well.

The immune system in mental disorders

Contrary to the popular myth of the brain as an "immune privileged organ", the nervous and immune systems are significantly interconnected. In fact, cytokines that are traditionally thought of as immune signaling molecules play important roles in neuronal functions as well (for review see (Bauer, et al., 2007)). For example, IL-6 modulates neuronal long term potentiation, a process thought to underlie learning and memory (Balschun et al., 2004; Jankowsky & Patterson, 1999). Cytokines can alter the morphology of several cell types of the brain; neurons retract their processes in response to application of different cytokines; astroglia and microglia can activate and take on a different morphology and function after immune stimulation (Bauer, et al., 2007; Gilmore, et al., 2004). On an organismal level, cytokines have been shown to mediate several behaviors. IL-6 and IL-1β are responsible for sickness behavior in mice, defined as the characteristic ruffling of fur, anhedonia, and suppression of appetite (Swiergiel,

Smagin, Johnson, & Dunn, 1997). These same cytokines probably cause the feeling of malaise associated with sickness in humans as well. Injection of LPS into human volunteers causes depression and anxiety-like symptoms (Reichenberg et al., 2001). IFN-α given to cancer patients to lessen the side effects of chemotherapy also causes depression (Raison, Capuron, & Miller, 2006). Clearly, cytokines have the potential to alter behavior in both the developing and adult brain (for review see (Yirmiya, 2000)).

It is possible that abnormal inflammation is a core feature of schizophrenia and autism, as there is evidence of ongoing immune activation in the brains of affected individuals. Immune dysregulation has been frequently reported in blood (Croonenberghs, Bosmans, Deboutte, Kenis, & Maes, 2002; Garver, Tamas, & Holcomb, 2003; Singh, Warren, Odell, & Cole, 1991; Zhang, Zhou, Cao, Wu, & Shen, 2005; Zimmerman et al., 2005), cerebrospinal fluid (Chez, Dowling, Patel, Khanna, & Kominsky, 2007; Zimmerman, et al., 2005) and brain (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005) from both schizophrenic (for review see (Drzyzga, Obuchowicz, Marcinowska, & Herman, 2006)) and autistic individuals (Table II). Moreover, microarray and proteome studies consistently show dysregulation of immune-related transcripts and proteins in schizophrenic(Arion, Unger, Lewis, Levitt, & Mirnics, 2007; Martins-de-Souza et al., 2009; Saetre et al., 2007) and autistic(Garbett et al., 2008) brains. The most visually compelling data supporting immune dysregulation is the severe glial activation reported in the postmortem autistic brain (Vargas, et al., 2005). Despite different ages, causes of death and co-morbid diseases (e.g., epilepsy) among subjects, a consistent histological pattern of dense, activated microglia (mainly in grey matter) and astroglia (mainly in white matter) was observed. A replication of the microgliosis was reported in autistic

brains as young as four years (Morgan, Chana, Buckwalter, Courchesne, & Everall, 2007).

Given the biological activity of cytokines, it is likely that this immune dysregulation plays an acute as well as developmental role in the behavioral abnormalities of patients. Parents report transient improvement in symptoms when an autistic child is sick (Curran et al., 2007), and the psychotic symptoms of schizophrenics given malaria are also reported to improve transiently (Hinsie, 1929; Tempelton & Glas, 1924). These types of sicknesses would alter the cytokine balance peripherally, and could therefore affect the brain's immune status as well (Dantzer & Kelley, 2007). In addition, there is a growing recognition that antipsychotic drugs can have anti-inflammatory effects (for review see (Drzyzga, et al., 2006)). For example, olanzapine, risperidone, perospirone, ziprasidone, quetiapine and aripprazole suppress LPS-induced NO production in microglial cells (Bian et al., 2008; Hou et al., 2006; Kato, Monji, Hashioka, & Kanba, 2007), and some of these drugs suppress pro-inflammatory cytokines in LPS-treated mice (H. Sugino et al., 2009). Perhaps the antipsychotic effects of these drugs are due to their anti-inflammatory properties as well as their antagonism of D2 dopamine receptors. In fact, several anti-inflammatory drugs have shown promise in the treatment of mental disease. Celecoxib, a COX-2 inhibitor, and minocycline, a tetracycline derivative with anti-inflammatory effects on microglia, have been reported to improve symptoms in schizophrenic patients (Akhondzadeh et al., 2007; Miyaoka et al., 2008). While the minocycline trial was open-label, the Celecoxib trial was double-blind and placebo-controlled. Pioglitazone (Actos©), also an anti-inflammatory drug that inhibits microglial activation, showed promise in an early trial in autistic patients (Boris

et al., 2007). Parents reported improvements in several core behaviors in 76% of 25 children enrolled. A double-blind, placebo controlled trial of minocycline for the treatment of autism is ongoing at the NIH. However, trials testing drugs in children are time-consuming, expensive and ethically delicate. Novel treatments based on anti-inflammatory therapies can initially be tested in the rodent models described here that have face and construct validity.

Mice born to dams that received immune-activating treatments during pregnancy do show increased levels of inflammation in both the periphery and the brain. As with cytokine expression in the fetus immediately following MIA, more severe immune-activating treatments tend to produce more severe ongoing inflammation in adult offspring. Daily injection with LPS throughout pregnancy produces offspring with chronically elevated levels of peripheral inflammatory cytokines. Treatment with antipsychotic drugs normalizes not only their behavior, but their cytokine profiles as well (E. Romero et al., 2007). Three injections of IL-6 into pregnant mice produced elevated levels of IL-6 in the serum of the adult offspring (Samuelsson, et al., 2006). These animal models demonstrate the power of antipsychotic drugs to dampen abnormal inflammation while altering behavior, and highlight MIA in rodents as a model for features of human psychiatric disease.

How does immune activation early in development set up an ongoing inflammatory state that persists months or years after the original insult? Does immune activation cause mental illness, or does a mis-wired brain cause inflammation? Ongoing research is just beginning to address these questions. In the meantime, analogies to other, more thoroughly-characterized model systems may guide our thinking.

One hypothesis is that the abnormal immune system precipitates abnormal behavior in the offspring. Maternal stress causes persistent behavioral, physiological and biochemical changes in the adult offspring. Maternal stress is a risk factor for schizophrenia (Beydoun & Saftlas, 2008), and a rat model was developed in the mid-1990s in which researchers stress pregnant rats by restraining them in a tube for several hours each day. The adult offspring displayed an elevated response to stress as adults (as well as other abnormal behaviors). When stressed themselves, the offspring of stressed mothers show a prolonged elevation of corticosterone after the stressor is removed compared to offspring of unstressed rats (Barbazanges, et al., 1996). A similar result was recently reported in people whose mothers were exposed to stress during pregnancy (Entringer, Kumsta, Hellhammer, Wadhwa, & Wust, 2009). This effect is dependent on corticosteroids, as stress in adrenalectomized pregnant rats does not affect the offspring, while corticosteroid injection does (Barbazanges, et al., 1996). Mechanistically, corticosteroid injections alter the level of glucocorticoid receptor (GR) in the hippocampus, an area important in terminating the stress response (Levitt, Lindsay, Holmes, & Seckl, 1996; Meaney et al., 1996). A related model involves the stress response of young rats whose mothers show high or low levels of pup-directed licking and grooming behavior (LG). High-LG offspring have a reduced stress response compared to low-LG offspring, with correspondingly different levels of GR. Maternal behavior alters GR expression in the offspring by altering histone acetyltransferase activity and subsequent methylation of the GR gene (for review see (Weaver, 2009)). By analogy, perhaps a similar developmental "homeostat" is set early in development based on normal, low-level cytokine exposure. In the MIA model where cytokines are

abnormally elevated during a critical period of development, levels of a receptor protein could be altered, perhaps via DNA methylation, leading to persistent changes to baseline cytokine levels.

An alternative hypothesis is that the abnormal brain causes an abnormal immune system. If, for example, inhibitory GABAergic interneurons fail to properly develop, the resulting brain circuitry would show an imbalance between levels of excitation and inhibition. Clinically, this may manifest as seizures (as observed in 40-50% of autistic children), or the loss of GABAergic markers such as parvalbumin (PV) (as is seen in schizophrenic brains (Beasley & Reynolds, 1997; Hashimoto et al., 2003), and offspring of MIA mothers (Meyer, et al., 2008)). Psychomimetic drugs such as ketamine, an NMDA antagonist, produce schizophrenia-like symptoms by inhibiting GABAergic neurons and producing a dis-inhibition of excitatory circuits (Behrens, Ali, & Dugan, 2008). In fact, loss of GABAergic signaling may be a core feature of schizophrenia (Lewis, Hashimoto, & Volk, 2005). Hyperexcited neuronal circuits can also produce an inflammatory state. Using a mouse model in which subchronic administration of ketamine induced a loss of PV-positive neurons, Behrens *et al.* (Behrens et al., 2007) demonstrated that ketamine caused long-term damage to PV neurons by activating the superoxide-producing enzyme NADPH oxidase (NOX). NOX production was, in turn, dependent on neuronal IL-6 produced in response to sustained hyperexcitability (Behrens, et al., 2008). This activation could be a homeostatic response to reduced inhibition whereby IL-6-dependent free radical production reduces excitatory transmission (via sensitivity of glutamatergic synapses to free radicals) and restores the inhibitory/excitatory balance of the circuit (Behrens, et al., 2008). However, prolonged

exposure to ketamine over-activates this feedback system, and the free radical production causes long-term damage to PV interneurons. Similarly, if IL-6 elevation due to MIA were to reach the fetus at a critical period of development, it could cause a reduction in PV interneurons, as reported in the MIA model (Meyer, et al., 2008). This loss of inhibition could create a hyper-excited circuit similar to a ketamine-exposed brain, leading to more IL-6 production by a mechanism similar to ketamine exposure. This IL-6 production would further suppress PV interneurons, creating more excitation and an ongoing positive feedback loop. Perhaps this process is set into motion soon after birth, resulting in autism, or perhaps a marginally stable circuit needs some later stimulus, such as peri-pubertal synaptic pruning, to tip the balance and create an inflammatory, hyperexcited, hallucinatory state (schizophrenia).

Recognizing the interrelatedness of the brain and the immune system allows us to look at mental illness differently. Emerging evidence includes the anti-inflammatory action of anti-psychotic drugs (and the potential efficacy of anti-inflammatory drugs as anti-psychotics), the behavioral consequences of exposure to inflammatory stimuli, and the profound dysregulation of the brain's immune status seen in mental illness. Whether an affected brain causes inflammation, or inflammation causes a brain to malfunction is an important issue in current research. Answers to these key questions could fundamentally alter how we diagnose, treat or even prevent mental illness.

Treatment	Findings	Reference
0.05 mg/kg LPS I.P. E18 rat	No change in TNFa, IL1b, IL6	(Ashdown et al., 2006)
0.12 mg/kg LPS I.P. E17 mouse	IL-6 increased	(H. Golan, Stilman, Lev, & Huleihel, 2006)
1 mg/kg LPS I.P. E18 rat	TNFa, IL-1b, iNOS increased *	(Paintlia, et al., 2004)
50ug LPS I.P. E18 mouse	IL-1b, IL-6, MCP-1, VEGF increased *	(Liverman et al., 2006)
2.5 mg/kg LPS I.P. E16 rat	TNFa increased	(Urakubo, Jarskog, Lieberman, & Gilmore, 2001)
4 mg/kg LPS I.P. E18 rat	TNFa, IL-1b increased *	(Cai, et al., 2000)
20 mg/kg poly(I:C) I.P. E16 rat	No change in TNFa	(Gilmore, Jarskog, & Vadlamudi, 2005)
2 mg/kg poly(I:C) I.V. E9 mouse	TNFa, IL-1b, IL-6, IL-10 increased	(Meyer, et al., 2007)
5 mg/kg poly(I:C) I.V. E9 mouse	IL-1b, IL-6 increased	(Meyer et al., 2006)
5 mg/kg poly(I:C) I.V. E17 mouse	IL-1b, IL-6, IL-10 increased	(Meyer, Feldon, Schedlowski, & Yee, 2006)

Table I. Maternal immune activation elevates cytokines in fetal brain. While some authors report no changes in cytokine levels, the majority of studies show significant increases. The studies that report no changes use less severe methods of immune activation (lower dose of LPS or I.P. administration of poly(I:C)). Assays are for cytokine protein, except where noted (*mRNA assayed).

Sample Analyzed	Findings	Reference
Serum from 23 autistic children	Increased IL-2 and T8 antigen	(Singh, et al., 1991)
Serum from 35 autistics	Increased TNFR II	(Zimmerman, et al., 2005)
Unstimulated whole blood culture supernatant from autistic children	Increased IFNγ and IL-1RA, IL-6 trended higher (p= 0.06)	(Croonenberghs, et al., 2002)
Unstimulated PBMCs culture supernatant from 10 autistic children	Increased TH2 cytokines, increased IL-13/IL-10 and IFNγ/IL-10 ratios	(Molloy et al., 2006)
Whole blood from 31 autistic children	Increased monocyte count, increased neopterin level	(Sweeten, Posey, & McDougle, 2003)
CSF from 12 autistic children	Increased biopterin, decreased quinolinic acid and neopterin	(Zimmerman, et al., 2005)
CSF from 10 autistic children	Increased TNFα	(Chez, et al., 2007)
CSF from 6 autistic children	Increases in many cytokines, including IFNγ, TGFβ2 MCP-1 IL-8	(Vargas, et al., 2005)
Fresh-frozen tissue from 7 autistic brains	Increases in many cytokines, including MCP-1, IL-6 and TGFβ1	(Vargas, et al., 2005)
LPS-stimulated PBMCs from 71 autistic children	Increased TNFα, IL-1β and IL-6 production	(Jyonouchi, Sun, & Le, 2001)

Table II. Inflammatory biomarkers are increased in autism. Studies of serum, blood cell cultures, cerebrospinal fluid (CSF), and brain tissue consistently find elevated levels of pro-inflammatory markers in autistic individuals. For a thorough review of similar findings in schizophrenia, see (Drzyzga, et al., 2006). PBMC, peripheral blood mononuclear cells.

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Chapter 4

Maternal immune activation yields offspring displaying the three core symptoms of autism

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Abstract

The core symptoms of autism are deficits in social interaction and language, and the presence of repetitive/stereotyped behaviors. We demonstrate that behaviors related to these symptoms are present in a mouse model of an environmental risk factor for autism, maternal infection. We stimulate the maternal immune system by injecting the viral mimic poly(I:C) during pregnancy, and analyze the social and communicative behaviors of the offspring. In one test, young pups respond to a brief separation from the mother with ultrasonic vocalizations (USVs). We find that, compared to pups born to saline-injected mothers, pups born to maternal immune activation (MIA) mothers produce a lower rate of USVs in the isolation test starting at day 8. The quality of the vocalizations is also different; analysis of sound spectrograms of 10 day-old pups shows that male pups from MIA mothers emit significantly fewer harmonic and more complex and short syllables. These communication differences are also apparent in adult offspring. Compared to controls, adult MIA males emit significantly fewer USVs in response to social encounters with females or males, and display reduced scent marking in response to female urine. Regarding a second autism symptom, MIA males display decreased sociability. In a third test of characteristic autism behaviors, MIA offspring exhibit increased repetitive/stereotyped behavior in both marble burying and self-grooming tests. In sum, these results indicate that MIA yields male offspring with deficient social and communicative behavior, as well as high levels of repetitive behaviors, all of which are hallmarks of autism.

Introduction

Autism is a neurodevelopmental disorder characterized by social impairments, communication difficulties and repetitive/stereotyped behaviors. While there is a substantial genetic component in autism, there is also evidence that environmental factors can contribute to the complex pathogenesis of autism spectrum disorders (ASD). Recent, large twin studies show a substantial concordance for autism between dizygotic as well as monozygotic twins ([Rosenberg et al., 2009] and [Hallmayer et al., 2011]). Moreover, the significantly higher risk for dizygotic twins compared to non-twin siblings suggests a role for the maternal environment. In fact, epidemiologic studies provide evidence that maternal exposure to stress, infection, anti-depressant medications, thalidomide or valproic acid (VPA) increases the risk for ASD in offspring (reviewed by [Hyman et al., 2005], [Kinney et al., 2008],[Patterson, 2009] and [Patterson, 2011a]). In particular, a recent study of over 10,000 records in the Danish Medical Register found a strong association between autism and maternal viral infection in the first trimester and a less robust but significant association between autism and maternal bacterial infection in the second trimester (Atladottir et al., 2010). There is also evidence of elevated levels of the chemokine MCP-1 in the amniotic fluid in ASD cases (Abdallah et al., 2012), and elevated levels of IFNγ, IL-4 and IL-5 in maternal serum are associated with increased risk for ASD in the offspring (Goines et al., 2011). These new results greatly extend prior work on the connection between maternal infection and autism.

Animal models displaying certain autistic features can be generated by maternal environmental challenges such as infection and exposure to VPA. Such models can be useful tools for testing epidemiologic findings and investigating the molecular

mechanisms underlying the neuropathology (reviewed by [Iwata et al., 2010], [Hsiao et al., 2011] and [Patterson, 2011b]). Deficits in communication are among the most prominent and functionally important symptoms in ASD, and acoustic and chemical signals are two primary modes of communication in rodents. The number and quality of USVs and the chemical composition of urine can convey information regarding health and fitness. Male responses to female urine can involve USVs and marking the surrounding territory (scent marking). A deficit in social interaction is also a critical symptom of ASD, and there are a number of paradigms used to assess this feature in rodents. One test that was originally introduced to assay social affiliation and pair bonding in voles utilizes a three chamber apparatus. In mice, this can include a test for preference for an unfamiliar mouse over an unfamiliar object (Silverman et al., 2010). The third cardinal symptom of autism involves repetitive/stereotyped behavior, which can be assayed in mice by quantifying self-grooming and compulsive burying of marbles placed in the nest (Thomas et al., 2009).

In the present study, we used these various assays to determine how closely the core symptomatology of autism can be reproduced in a model of an environmental risk factor for ASD. This model involves activating the immune system of pregnant mice in order to mimic the effects of maternal infection. Young and adult offspring of these mice were assessed for relevant behaviors in several paradigms.

Results

Pups born to MIA mothers display a deficit in the number of USVs and an altered syllable repertoire

Neonatal rodents emit USVs when separated from their mothers or littermates in order to attract the mother for retrieval to the nest. In most mouse strains, the rate of pup USVs increases during the first 5–6 postnatal days, reaching a peak on days 8–10, and decreasing to zero by the end of the second postnatal week (Motomura et al., 2002). We evaluated male MIA and control offspring for USV production in the isolation test during second postnatal week. Compared to controls, male pups from MIA mothers emit fewer vocalizations ($F(1, 56) = 17.74$, $p < 0.0001$; Bonferroni posthoc test $p < 0.05$ for P8, P10 and P12 pups) and the total vocalization duration is less starting from day 8 ($F(1, 56) = 15.74$, $p < 0.0005$; Bonferroni posthoc test $p < 0.05$ for P8, P10 and P12 pups) (Fig. 1A and B).

In order to minimize the effect of repetitive handling and isolation on pup USVs, data for structural analysis of the USVs were collected from pups tested only on P10. The proportion of each syllable type is shown in Fig. 1C. Since there is no difference in the proportion of one, two and multiple frequency step syllables, we present them as one group termed frequency step syllables. Although a two-way ANOVA test does not indicate a difference in the distribution of USV syllables between treatment groups (treatment, $F(1, 41) = 0.292$, $p = 0.58$), significant effects of syllable type ($F(8, 180) = 79.97$, $p < 0.0001$) and treatment × syllable type interaction ($F(8, 180) = 3.67$, $p < 0.0006$) are evident. However, the Bonferoni post-hoc test shows a treatment-dependent effect on the proportion of syllables in three of the nine syllable types. Compared to controls, MIA offspring produce 60% fewer harmonic syllables ($p < 0.05$)

and significantly more short (MIA 13% versus control 7%, $p < 0.05$) and complex syllables (MIA 13% versus control 9%, $p < 0.05$) (Fig. 1C). ANOVA analysis of the duration of each syllable type also shows a difference between MIA and control offspring (treatment, $F(1, 41) = 4.32$, $p < 0.05$). The Bonferoni post-hoc test reveals a difference between the treatment groups in complex, downward and upward syllables ($p < 0.05$) (Table 1). However, no difference is found in the dominant frequency of any of the USV syllables (treatment, $F(1, 41) = 0.022$, $p = 0.88$ (Table 2).

Body weight was used as a parameter to evaluate animals' health and it does not differ between these two groups (Fig. S3). Moreover, we find no changes in the development of reflexes (negative geotaxis, grasp reflex and righting reflex) (data not shown). In addition, we analyzed maternal behavior toward pups in response to either nest relocation or to pup retrieval and found no difference between two treatment groups (Fig. S4). Thus, the reduction in the USV production is not due to a delay in gross pup development or evident differences in maternal responsiveness toward the offspring.

Adult male offspring from MIA mothers display reduced USV responses to various social stimuli

Adult mice produce USVs in several social contexts (Portfors, 2007). Encounters with females induce male USV production, which is most prominent during initial investigatory behavior and correlates well with the level of male sexual arousal (White et al., 1998). This USV production conveys an important communicative function, predominantly to engage the female's attention. Before studying male–female interactions, we used anesthetized animals to conduct a pilot experiment to evaluate the

contributions of male and female mice in USV production. We detect USVs in two settings – when both the male and female are awake and when a male is exposed to an anesthetized female. In contrast, few vocalizations (2–3 during 3 min testing period) are detected when the male is anesthetized, although the female mouse interacts physically with the male by sniffing and walking around him (data not shown). Thus, female mice do not contribute significantly in USV production, at least in our test setting. Similar results were reported by several research groups using devocalized or anesthetized males ([Maggio and Whitney, 1985], [White et al., 1998] and [Sugimoto et al., 2011]). To test vocalizations in adult MIA offspring, we introduced an unfamiliar female to the male and recorded USVs. We find that, compared to control males, MIA offspring produce significantly fewer vocalizations in the presence of a female ($t(41) = 2.092$, $p < 0.05$) (Fig. 2A). While analysis of USV syllables using the two-way ANOVA does not show a significant difference in the distribution of syllable types between MIA and control males (treatment, $F(1, 41) = 1.68$, $p = 0.2$; syllable type, $F(10, 450) = 54.66$, $p = 0.0001$; treatment × syllable type, $F(10, 450) = 1.13$, $p = 0.3$), the Bonferoni post-hoc test reveals a significant reduction in the MIA offspring in the number of two frequency step (MIA 8% versus control 12%, $p < 0.05$) and chevron syllables (MIA 10% versus control 13%, $p < 0.05$) (Fig. 2B). The two-way ANOVA test shows a difference between MIA and control male offspring in duration and dominant frequency of USV syllables during interaction with females (treatment, $F(1, 41) = 7.42$, $p < 0.05$ for syllable duration and $F(1, 41) = 9.4$, $p < 0.05$ for dominant frequency of syllables). The Bonferoni post-hoc test of individual syllables reveals a significant difference in duration of three and more

frequency step syllables ($p < 0.05$) (Table 1 and Table 2). As with the pups, no treatment difference is found in the dominant frequency of any USV syllables (Table 2).

Before studying male–male interactions, we also conducted a pilot experiment to evaluate the contributions of resident and intruder mice in USV production using anesthetized animals. We detected USVs in only two settings, when both males were awake or when the intruder was anesthetized. In contrast, no vocalizations were recorded when the resident was anesthetized, although the intruder mouse interacted physically with the resident by sniffing and licking him (data not shown). The pilot experiment suggests that resident males respond to an encounter with a male intruder by producing USVs, which likely serve an important defensive function. We next analyzed the responses of adult male offspring of control and MIA mothers to the presence of an unfamiliar male intruder. Compared to controls, MIA offspring produce significantly fewer USVs during this social encounter (Mann–Whitney $U = 99$, $p < 0.05$ one-tailed) (Fig. 3A). Since not all tested males produced USVs in the presence of a male intruder, the number of samples used to analyze the sound spectrograms was 16 for control and 19 for experimental groups. While the two-way ANOVA test does not an effect of treatment on the total distribution of all syllables for male-male interaction (treatment, $F(1, 33) = 3.31$, $p = 0.08$; syllable type, $F(10, 330) = 84.04$, $p = 0.0001$; treatment × syllable type, $F(10, 330) = 0.83$, $p = 0.8$), the Bonferoni post-hoc test of individual syllable differences reveals a significant reduction in the number of two frequency step syllables emitted by the MIA offspring (MIA 5% versus control 8%, $p < 0.05$) (Fig. 3B). No difference is seen in the duration of any USV syllables (treatment, $F(1, 41) = 2.4$, $p = 0.12$) (Table 1). Two-way ANOVA test shows a difference between MIA and control

male offspring in the dominant frequency of all USV syllables during male–male interaction (treatment, $F(1, 41) = 16.32$, $p < 0.001$). However, the Bonferoni post-hoc test reveals a significant change in the dominant frequency for flat syllables only ($p < 0.05$) (Table 2). Thus, in all three social paradigms, there is a decrease in the number of USVs emitted by the MIA offspring. In both of the adult social paradigms, MIA offspring produce fewer two frequency step syllables. Our results indicate that MIA not only affects USV production by the offspring, but also changes the quality of their communication in several social settings that have important biological roles in survival and reproduction.

MIA males display a communication deficit in female urine-induced scent marking

In addition to communication using acoustic signals, mice gain information through olfaction. Scent marks (urinary traces) are used to determine mouse identity, mark territory, and to attract mates (Hurst, 2009). During the 60-min habituation to a new clean cage, the two groups of mice showed no difference in scent marking. The total density of urine deposits for control and experimental males were 3899 ± 378 and 4224 ± 525 for control and experimental groups, respectively; ($t(41) = 0.51$, $p = 0.615$). Then we used fresh male or female urine to stimulate scent marking from adult male mice in order to assess olfactory communication. Males from both MIA and control groups spend equal time exploring a female urine spot (data not shown), suggesting that they are both processing the olfactory social stimulus. (A further test of olfactory sensitivity is described below). But male offspring from MIA mothers deposit half as many scent marks compared to control animals during the 3 min test period

($t(41) = 3.266$, $p < 0.005$) (Fig. 4A and Fig. S5). Interestingly, no group difference is found when male urine is used as the stimulus ($t(41) = 0.021$, $p = 0.98$) (Fig. 4B). It should be noted, however, that in control males, male urine induces half as many scent marks as does female urine ($t(40) = 4.434$, $p < 0.0001$). In sum, MIA males display a communication deficit in female-induced scent marking, which plays a key role in mediating sexual behavior.

MIA offspring display reduced sociability

Social behavior is a form of communication between members of the same species, and this type of behavior in rodents is thought to constitute a reasonable model of human interpersonal interaction. The three chamber apparatus can be used to assess autism-related behaviors such as sociability (Silverman et al., 2010). Shortly after a period of habituation, sociability is evaluated as the time the subject mouse spends in the chamber containing a wire cage holding an unfamiliar mouse compared to the time spent in the opposite chamber containing an empty wire cage (novel object). We find that males from saline-injected mothers spend 65% of the time in the social chamber and 26% of the time in the chamber with the novel object. In contrast, offspring of MIA mothers display decreased preference for the social chamber, spending only 50% of the time exploring the social chamber and significantly more time (37%) than controls exploring a novel object ($F(2, 48) = 12.73$, $p < 0.0001$; Bonferroni posthoc test $p < 0.001$ for time in the social chamber and $p < 0.01$ for time in the chamber with novel object) (Fig. 5A). This results in significantly different ratios of time spent in the two compartments (preference index) compared with the offspring from saline-injected mothers

($t(41) = 3.283$, $p < 0.005$; Fig. 5B). This result is similar to that obtained with offspring of mothers injected just once with poly(I:C), and where both sexes were tested (Smith et al., 2007).

MIA offspring display high levels of repetitive/compulsive behavior in the marble burying test

The marble burying test presents a novel situation that can evoke a highly repetitive and stereotyped response. The marble burying test was previously used to evaluate anxiety but it was recently shown that this behavioral response does not correlate with results from other tests of anxiety. Thus, marble burying is now used to evaluate repetitive and perservative behavior in small rodents (Thomas et al., 2009). We find that the offspring of MIA mothers display extremely high repetitive behavior in this test ($t(41) = 8.535$, $p < 0.0001$) (Fig. 6A and Fig. S6). Compared to the control group, MIA offspring bury 2.8-fold more marbles during the testing period.

MIA offspring display elevated levels of self-grooming

Self-grooming can provide another index of repetitive/stereotypic behavior in rodents (Silverman et al., 2010). We tested individual mice in a small glass beaker for 10 min, as it is known that a restricted environment induces repetitive behavior (Lewis et al., 2007). MIA male offspring spend 50% more time on self-grooming than controls ($t(41) = 2.335$, $p < 0.05$) (Fig. 6B).

MIA offspring do not display an obvious olfactory deficit

Since MIA offspring display decreased sociability and communication, we evaluated their olfactory sensitivity. Time spent sniffing a novel, vanilla-scented paper versus a plain water-scented paper was recorded, and no group difference is evident ($t(18) = 0.361$, $p = 0.72$) (Fig. 7). Therefore, the social and communication deficits found in the MIA offspring are not likely to be caused by olfactory malfunction.

Discussion

We here show that the offspring of poly(I:C)-treated mothers exhibit the three core features of autism. While prior work from our laboratory had demonstrated deficits in social interaction in MIA offspring, those studies utilized a different poly(I:C) injection schedule, different methods, and did not analyze males specifically ([Shi et al., 2003], [Smith et al., 2007] and [Hsiao and Patterson, 2011]). The present study is also the first to analyze scent marking, USVs, and repetitive/stereotyped behaviors in the MIA model. Moreover, this is the only model of an environmental risk factor for autism in which all three of the core features have been analyzed thus far.

In the model of a different environmental autism risk factor, maternal VPA, behavioral studies revealed two core autism symptoms, repetitive/stereotypic-like hyperactivity and impairment in social interaction ([Schneider and Przewłocki, 2005] and [Schneider et al., 2006]). In the rat model of maternal stress, there are also male-specific behavioral abnormalities: poor sociability (Lee et al., 2007) and sexual behavior (Ward and Reed, 1985), as well as deficits in pup USVs ([Morgan et al.,

1999] and [Zimmerberg and Blaskey, 1998]). Activation of the maternal immune system with lipopolysaccharide (LPS), which mimics bacterial infection, also yields offspring with decreased social interaction ([Hava et al., 2006] and [Kirsten et al., 2010]). In addition, pups from LPS-injected mothers display impaired play behavior and USV communication ([Hava et al., 2006], [Baharnoori et al., 2010] and [Kirsten et al., 2010]). A neuropathology characteristic of autism, a spatially localized deficit in Purkinje cells in the cerebellum, is also found in the VPA and MIA mouse models ([Ingram et al., 2000] and [Shi et al., 2009]). Since the cerebellum is known to contribute to learning, language, sociability and emotionality ([Ito, 1998] and [Thach, 1998]), the deficits in communication and social interaction observed in MIA and VPA mice may be associated with abnormalities in the cerebellum as well as other brain regions. Together, these rodent models exhibit a spectrum of autistic-like behaviors.

It should be noted that maternal infection models also display features of schizophrenia. These include enlarged ventricles, enhanced responses to amphetamine and hallucinogens, alterations in dopamine and serotonergic pathways, as well as a number of other behaviors in the offspring that are also found in subsets of autistic subjects such as enhanced anxiety and eye blink conditioning, as well as a PPI deficit ([Iwata et al., 2010], [Hsiao et al., 2011], [Meyer and Feldon, 2010], [Patterson, 2011b] and [Moreno et al., 2011]). This is not surprising, as maternal infection is a well-characterized risk factor for schizophrenia as well as autism (Brown and Derkits, 2010). Moreover, these disorders have symptoms in common, such as impaired social interaction, elevated anxiety and abnormal responsivity to stress (Tordjman et al., 2007). On the other hand, schizophrenia and autism obviously have symptoms that differentiate them. Autism occurs in early

childhood whereas the psychotic symptoms of schizophrenia appear much later. In addition, delusions and hallucinations are typical of schizophrenic patients while motor stereotypies and lack of language are cardinal symptoms of autism. Thus, the MIA model displays both symptoms in common between these two disorders, as well as symptoms that differentiate them. Presumably the different disease phenotypes associated with maternal infection in human autism and schizophrenia are due to differences in genetic background of the mother and/or fetus, the timing of the infection, the presence of allergies or autoimmune disease in the mother, and possibly the severity of the infection.

The present study shows that MIA offspring display altered emotional behavior early in postnatal life. The male pups display a reduced number of vocalizations when they are isolated from their littermates and mother. Similar findings were reported for pups born to mothers exposed to stress or LPS during pregnancy ([Morgan et al., 1999] and [Baharnoori et al., 2010]), and in reelin, Mecp-2 and CNTNAP2 mutant mice ([Ognibene et al., 2007], [De Filippis et al., 2010] and [Peñagarikano et al., 2011]). The reduction in USV production is not, however, due to a delay in gross pup development or a difference in maternal care. Altered USVs can reflect a reduced response to stress or poor bonding with the mother; the latter was characterized in mice lacking the μ-opioid receptor gene (Moles et al., 2004). The former explanation appears unlikely in the MIA model as we do not see any changes in the pup locomotor activity (circling and pivoting) during the isolation test (data not shown). Interestingly, BTBR pups, a mouse strain with several autism-like features, vocalize more in this test compared to several other strains of mice, which could be due to an elevated emotional response to the novel environment (Scattoni et al., 2008). In addition, the BTBR pups exhibit an altered vocal repertoire,

producing a high level of harmonics but minimal syllables with a simple structure (chevron-shaped, upward, downward and short). MIA pups also show some structural changes in their vocalization patterns, but these are opposite what was reported for BTBR pups: lower level of harmonic syllables but increased level of complex and short syllables. Harmonic syllables are known to be prominent in the mouse pup repertoire (Grimsley et al., 2011). They amount to 16% of the pup repertoire, whereas the proportion of this type of syllable is very low in adult male song during male–female and male-male encounters (Figs. 1C, 2B and 3B). It is also interesting that a short reunion with the mother affects pup emotional response to a second isolation as evidenced by increased USV production and augmented proportion of multi-component syllables including harmonics (data not shown; Young et al., 2010). Moreover, harmonic syllables have some similarity to human baby cries (Zeskind et al., 2011), which is known to reflect the emotional state of the baby. Thus, harmonic syllables can be used as an index of pup emotional status, and MIA offspring demonstrate a reduced emotional response to separation from the mother.

Importantly, we find that adult MIA males demonstrate decreased USV responses in social encounters with females or other males. Several mouse strains suggested as being relevant for autism studies (BTBR, NL-3-knockout (KO), NL-4 KO and Shank1 KO) show a similar deficiency ([Jamain et al., 2008], [Radyushkin et al., 2009], [Scattoni et al., 2011] and [Wohr et al., 2011b]). Frequency step syllables are the most frequent in adult male repertoire in both social settings (37–39%), and they likely reflect affiliative/social communication. Sound spectrogram analysis of MIA male vocalizations reveals reduced use of two frequency step syllables in both social paradigms. BTBR

males also emit fewer frequency step calls during interaction with females or males, although no changes in USV repertoire were found for NL-3 KO and NL-4 KO mice. Given the differences in syllable preference displayed by MIA versus control males, it will be of interest to examine such preferences more closely over developmental time, and in other social paradigms such as juvenile play. It will also be worthwhile to examine the serial order and groupings of the syllables to determine if these parameters are correlated with social paradigm, age or maternal insult. Such analyses could provide important information as to the relevance of syllable use in mice to the language deficit in autism. The deficit in male USVs in the presence of a female in estrous and the deficit in scent marking in the presence of female urine could suggest a lack of sexual interest. We know of no data on actual mating behavior or successful copulation by adult male MIA offspring. Similarly, the deficit in USVs in the presence of a male intruder suggests a lack of aggressiveness in defense of territory. It will be of interest in the future to test MIA males for dominant/submissive behaviors as well as sexual behavior.

In addition to deficits in auditory communication, MIA males exhibit a reduction in female urine-induced scent marking, which was also found in BTBR and Shank1 KO males ([Wohr et al., 2011a] and [Wohr et al., 2011b]). The deficits in auditory and olfactory communication tested here would also seem relevant for social interaction. The sociability test we used seems more appropriate for the social deficit in human autism than the social preference or social habituation tests. The latter tests are based on memory of prior social encounters, which has not been shown to be a key feature in autism social pathology. In contrast, the sociability test assesses whether the mouse prefers to be in the presence of a novel mouse versus a novel object. While many factors may be involved in

this decision (e.g., fear, curiosity, aggression), it does seem analogous to many human situations that confront autistic individuals. In this light, prior work on interaction with novel objects is of interest. MIA offspring display a deficit in interaction with a novel object (Shi et al., 2003), which is suggestive of anxiety in a novel situation, as is seen in autism. Further work of this type revealed that, compared to controls, MIA offspring display a distinct c-Fos expression pattern in hippoocampal area CA1 following novel object, but not novel location, exposure. Thus, the offspring of MIA mothers may have an abnormality in modality-specific information processing. Indeed, MIA offspring display enhanced discrimination in a novel object recognition, but not in an object location task (Ito et al., 2010). Thus, analysis of object and spatial information processing at both synaptic and behavioral levels reveals a largely selective abnormality in object information processing in this mouse model. We find that MIA offspring exhibit highly repetitive behavior in marble burying and self-grooming tests. Self-grooming is also increased in several other mouse strains with relevance to autism (BTBR, and Fmr1, MeCP2 and CNTNAP2 KO mice) ([McNaughton et al., 2008], [McFarlane et al., 2008], [Chao et al., 2010] and [Peñagarikano et al., 2011]).

In sum, this study provides new evidence that environmental risk factors such maternal infection during pregnancy yield the offspring with autistic-like behaviors, including communication and social interaction impairments as well as high repetitive behavior. This reinforces prior results indicating the face and construct validity of the model for both autism and schizophrenia.

Methods

Generation of animals

Female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were obtained from the California Institute of Technology breeding facility and were housed in ventilated cages under standard laboratory conditions. Mice were mated overnight, and the presence of a vaginal plug marked that day as embryonic day 0.5 (E0.5). Pregnant females were not disturbed, except for weekly cage cleaning, until E10.5 when they were weighed and pseudo-randomly assigned to one of two groups, poly(I:C) or saline. Each group initially contained five or six pregnant females. The maternal poly(I:C) treatment did not alter litter size (8.67 ± 0.56 and 7.83 ± 0.54 for control and experimental groups, respectively; $t(10) = 1.07$, $p = 0.309$). All pups from a single litter remained with the mother until weaning at P21, at which time male mice were housed with same-sex littermates in groups of two to four. All experimental mice underwent the same sequence of behavioral tests at the same times during development (n = 21 (5 litters) and 22 (5 litters) for control and experimental groups, respectively). All behavioral tests were conducted in behavioral testing rooms between 09.00 and 17.00 h during the light phase of the circadian cycle. Mice were habituated to a testing room at least 1 h prior to the start of the behavioral test. Order of testing was: (1) Pup USVs during the isolation test at age 10 days; (2) PPI at age 6 weeks (Supplemental materials); (3) open field at 7 weeks (Supplemental materials); (4) adult male USV responses to female and male stimuli at age 8–9 weeks; (5) three chamber social test at 10 weeks; (6) scent marking at 11 weeks; (7) marble burying at age 12 weeks; (7) self-grooming at age 13 weeks; and (8) olfactory sensitivity at age 13–14 weeks. A separate experiment was carried out with another cohort of mice (n = 29 (6

litters) and 29 (6 litters) to test the USV profile during the second postnatal week. This was done for two reasons: first, to minimize effect of handling and stress on the USV structure; second, to lessen the effect of repeated stress during isolation in early postnatal life on adult behavior.

Maternal administration of poly(I:C)

One group of mice was given intraperitoneal injections of 5 mg/kg poly(I:C) (potassium salt; P9582; Sigma, St. Louis, MO) or saline on E10.5, 12.5 and 14.5. The manufacturer supplies poly(I:C) at 10% of the total weight of the salt, and the dosage was based on the weight of poly(I:C) itself. The inflammatory cytokine response in pregnant mice induced by an acute systemic challenge of poly(I:C) is time-limited, and the time of maternal immune challenge influences the pattern of behavioral abnormalities in the offspring (Meyer et al., 2006). Therefore, we challenged the maternal immune system three times during pregnancy to determine if the offspring develop a broader range of behavioral deviances. However, where comparisons are possible, the behavioral results from the current offspring do not appear to be significantly different from our prior results using a single poly(I:C) injection on E12.5. Mice born to triple-injected mothers have the deficit in prepulse inhibition (PPI) and reduced locomotion in open field (Fig. S1) that we have seen in single-injected mothers and influenza-infected mothers ([Shi et al., 2003], [Smith et al., 2007] and [Hsiao and Patterson, 2011]).

Pup USVs during the isolation test

Pups from mothers injected with saline or poly(I:C) were tested for USVs every other day from day 6 to day 14 in the isolation test as described (Hofer et al., 2002). During the test, the dam was removed from the home cage and placed in a separate cage away from the litter. Fifteen minutes after removing the dam, male pups were individually removed from the nest in random order and gently placed into an empty 15 × 15 cm white Plexiglas box. Ambient temperature in the room was 23.5–23.6 °C. In the pilot experiment the pups' axillary temperature was measured prior and after testing and found to be decreased after isolation from 34.43 ± 0.19 to 31.97 ± 0.29 °C in 6 day-old pups. In a pilot experiment we tested P6 pups from MIA and control mothers in the isolation test and found no difference in the temperature decrease between the groups (data not shown). As it is known that small decreases in pup core temperature in the home cage before testing have no significant effect on their USV isolation performance (Hofer et al., 2002), we did not measure the body temperature in order to minimize the effect of handling on pup behavior. USVs were recorded for 3 min using an UltraSoundGate Condenser Microphone (CM 16; Avisoft Bioacoustics, Germany) suspended 7 cm from the testing surface. The microphone was sensitive to frequencies of 0–250 kHz with a flat frequency response (±6 dB) between 25 and 120 kHz. It was connected via an Avisoft UltraSoundGate 116 USB Audio device (Avisoft Bioacoustics) to a personal computer, and the recordings were carried out with a sampling rate of 250 kHz. The vast majority of USVs were detected within the 0–150 kHz range. The recordings were analyzed using Avisoft SASLab Pro software (see below). After recording, the pups were weighed and placed back into the nest. Body weight was included as an indicator of pups' nutritional

status and maturational age. The mother was returned to the pups after they all had been tested. The testing box was cleaned with water after each pup recording.

Adult male USV responses to female and male stimuli

Eight-to-ten week-old males were separated and housed singly for a week before testing. To provide a standardized history of sexual experience that is known to facilitate USVs (McGinnis and Vakulenko, 2003), adult male subjects were exposed to unfamiliar adult females for 15–20 min every day for 5 days. On the day of testing, the male was placed in a clean cage (30 × 16 × 14 cm) for 10 min habituation. The male was then tested for USVs in the presence of an unfamiliar female in the estrous phase for 3 min. The female was scored as in estrous when the vaginal area appeared wide, open and red. USVs were recorded as described above. After 5 min rest following removal of the female, the male subject was tested for USVs in the presence of an unfamiliar male intruder for 3 min. During male–male encounters, host males show characteristic behaviors such as active approaching, sniffing and following the intruder. USVs were analyzed using Avisoft SASLab Pro software as described below.

Analysis of USVs

For acoustical analysis, recordings were transferred to Avisoft SASLab Pro (version 4.38; Avisoft Bioacoustics) and a fast Fourier transformation was conducted (512 FFT-length, 100% frame, Hamming window and 75% time window overlap). Detection of USVs was provided by manually setting a threshold-based algorithm and a hold-time mechanism (hold time: 5 ms). Since no USVs were detected below 30 kHz, a lower cut-off frequency

of 30 kHz was used to reduce background noise outside the relevant frequencies. The accuracy of detection was verified by an experienced investigator. The total number of vocalizations emitted, total vocalization time as well as duration and dominant frequency for each syllable type were measured automatically. Spectrographic analysis was conducted as originally described by Scattoni et al. (2008) with some modifications that were also made by Grimsley et al. (2011). All USVs were assigned to one of 11 structural groups, primarily according to their pitch contour shape, which is determined by the frequency modulation and duration of the acoustic signal (Fig. S2). Syllables that were described as composite by Scattoni et al. (2008) were classified as harmonics because these syllables often had a third harmonic component at 150 kHz. If the syllable was compound and contained multiple components such as harmonic and frequency steps, it was classified as a frequency step syllable with a harmonic component. In a pilot experiment, we compared the distribution of USV syllables collected during the entire 3 min testing period and from every other 30 s period during the same 3 min testing and found that both methods of analysis yield similar data (Fig. S3). Thus, for the further analysis, USVs were collected from every other 30 s period during the 3 min testing period (average number of vocalizations per mouse was 250–300) unless an animal produced fewer than 100 vocalizations, in which case USVs were collected from the total 3 min of testing. The total number of USV syllables was normalized to 100%.

Three chamber social test

Ten week-old mice were tested in a 60 × 40 cm Plexiglass box divided into three chambers as described previously (Smith et al., 2007). Mice could freely move between

chambers through a small opening (6 × 6 cm) in each divider. Mice to be tested were placed in the center chamber, and an overhead camera recorded their movements. Mice were allowed 10 min to explore the empty box in order to evaluate bias for either of the side chambers. For each animal, we confirmed that there was no side bias. Then an unfamiliar, same-sex mouse was placed in a wire cage (11 cm height, 10.5 bottom diameter, bars spaced 1 cm apart; Galaxy Cup, Spectrum Diversified Designs, Inc., Streetsboro, OH) in one side chamber. Another empty wire cage was placed in the opposite side chamber as a non-social object. Mouse behavior was recorded for 10 min. The time spent in each of the three chambers was measured, and the preference index for social object was calculated as $[time_{social}/(time_{social} + time_{non\text{-}social})] \times 100 - 50$ (Jamain et al., 2008). All testing chambers were cleaned vigorously with 70% ethanol followed by Process NPD before and after testing each mouse.

Scent marking test

This test was done according to the protocol described by Wohr et al. (2011a). Subject mice were placed in a clean cage (30 × 16 × 14 cm) containing a sheet of absorbent paper that covered the entire base (Strathmore Drawing Paper Premium, 400 series; Strathmore Artist Papers, Neenah, WI, USA). Female urine was collected from adult C57/B6 females in estrous. Male urine was collected from sexually experienced adult C57/B6 males. The adult male subject was habituated for 60 min to the clean cage lined with fresh paper. After the habituation period, the mouse was placed back in the home cage. A new sheet of paper was then placed in the cage and 15 μL of female urine was aliquotted onto the center. The mouse was placed back in the testing cage for 3 min. Scent marks deposited

on the paper during habituation or exposure to female/male urine were visualized using 0.5% ninhydrin in 95% ethanol. Developed papers were scanned and analyzed for total density using ImageJ (NIH, USA). The data are shown as the ratio of density of the experimental group to the control group.

Marble burying test

This test was done as described by Thomas et al. (2009) with small modifications. In a pilot experiment under these conditions, we found that naive C57 males bury 50% of the marbles in a 10 min period so this testing time was used in further experiments so as to be able to detect either increases or decreases in the number of buried marbles. Clean cages (27 × 16.5 × 12.5 cm) were filled with a 4-cm layer of chipped cedar wood bedding. Males were habituated to this cage for 10 min and then returned to the home cage. Twenty navy blue glass marbles (15 mm diameter) were gently laid on top of the bedding, equidistant from each other in a 4 × 5 arrangement. Animals were placed back into the testing cage and the number of marbles buried (>50% marble covered by bedding material) in 10 min was recorded.

Self-grooming test

Self-grooming was evaluated in a 6.5 cm diameter × 10 cm tall, clear glass beaker covered with a filter top. After 10 min habituation in the beaker, self-grooming was measured for a further 10 min. The investigator sat 1 m away from the apparatus and recorded cumulative time spent in grooming (scratching fur with any foot). All testing

beakers were cleaned vigorously with water followed by Process NPD before and after testing.

Olfactory sensitivity test

The method for measuring olfactory sensitivity was adapted from Witt et al. (2009). On day 1, the subject was habituated to a clean empty cage for 10 min, and then a 2 × 2 cm damp Stathmore paper was placed in the cage and the time spent exploring the paper during 3 min period was recorded. On day 2, the paper was dampened with 10% vanilla scent in water instead of plain water. The data are presented as the time spent exploring the scented paper minus the time spent exploring the plain water paper.

Statistical analysis

Statistical analyses were carried out with Prism 4.0b software. Normally distributed data were plotted in the figures as means ± SEM. The means of two groups were compared by Student's t test. Where data were not normally distributed (assessed by the Kolmogorov–Smirnov test), they were plotted as medians plus 25% and 75% quartiles, and range. Two groups of such data were compared with the Mann–Whitney test. All statistical tests were two-tailed, except the number of vocalizations in the male-male encounter, with $\alpha \leqslant 0.05$. The differences among groups with two variables were assessed using two-way ANOVAs followed by Bonferroni posthoc tests. Significant differences emerging from the above tests are indicated in the figures by $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.005$.

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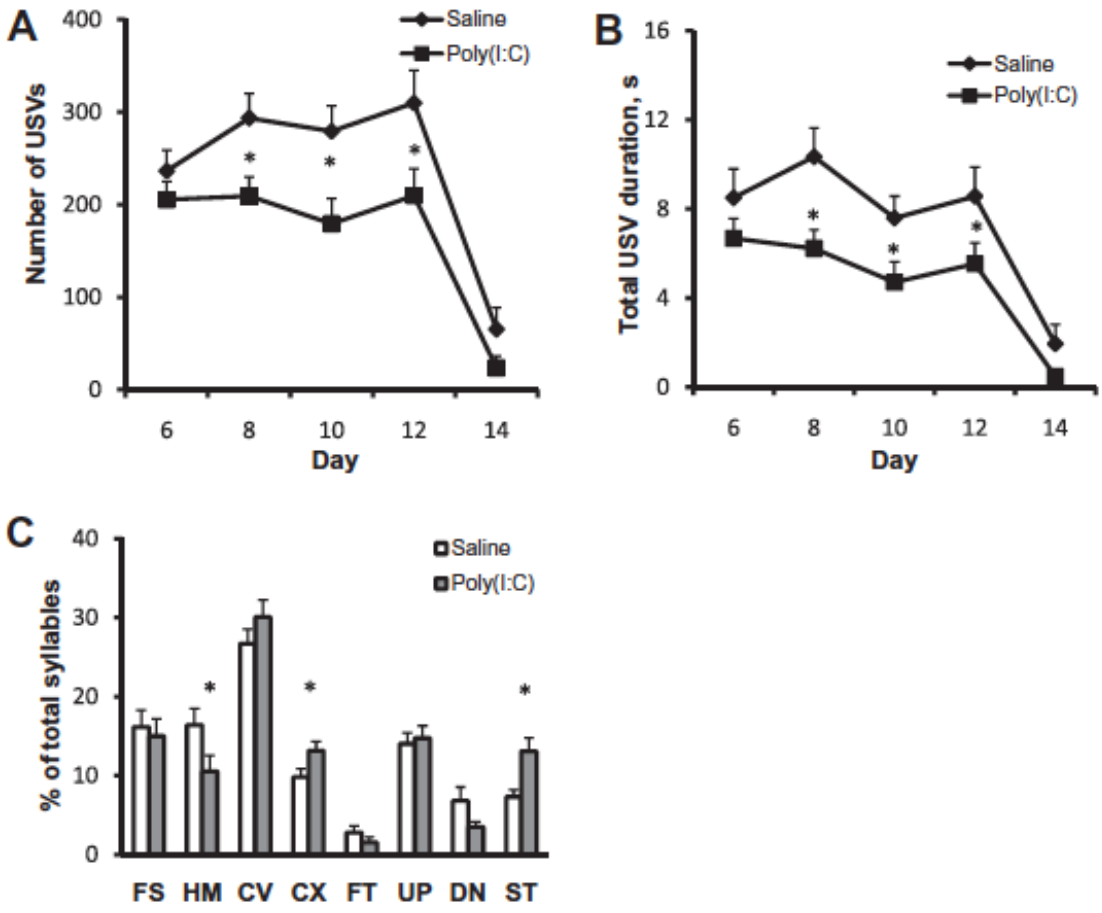

Fig. 1. Male pups from MIA mothers emit altered number and quality of USVs in the isolation test. Pups were isolated from the nest and tested individually for USVs every other day from day 6 to day 14. Compared to control pups, the MIA pups emit fewer vocalizations (A), call for a shorter duration of time (B). Ten day-old pups from MIA mothers also produce significantly more short and complex syllables and fewer harmonic syllables than controls. (C) Significant differences are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. USV classification: FS, frequency step; HM, harmonic; CV, chevron; CX, complex; FT, flat; UP, upward; DN, downward; ST, short; RC, reversed chevron.

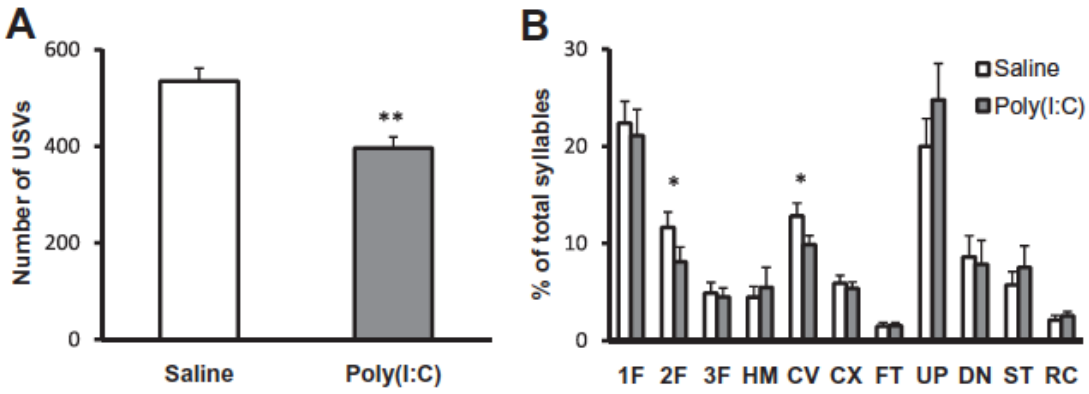

Fig. 2. Adult male offspring from MIA mothers display reduced USV responses in a social encounter with a female. During interactions with a female in estrous phase, MIA males emit fewer vocalizations (A) and also produce significantly fewer two step frequency and chevron syllables (B) than controls. Significant differences are indicated by *$p < 0.05$, **$p < 0.01$ and ***$p < 0.005$. USV classification: 1F, one frequency step; 2F, two frequency step; 3F, three and more frequency step; HM, harmonic; CV, chevron; CX, complex; FT, flat; UP, upward; DN, downward; ST, short.

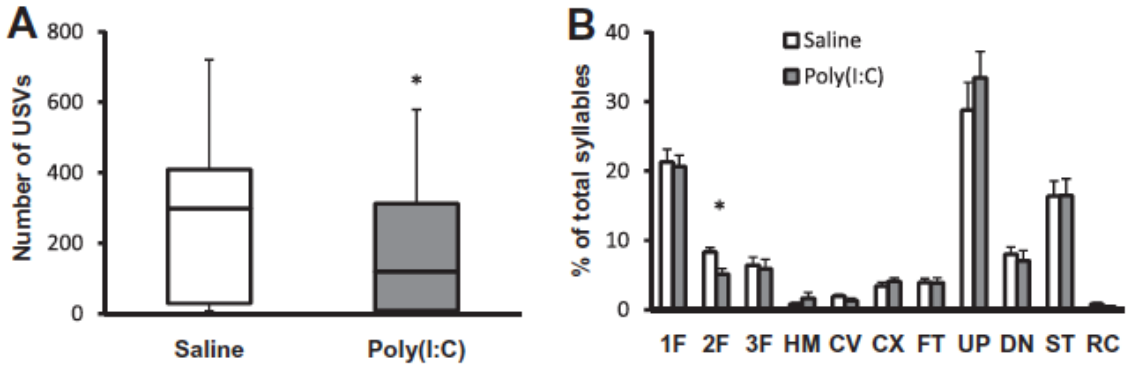

Fig. 3. Adult male offspring from MIA mothers display reduced USV responses in a social encounter with a male. During interactions with an unfamiliar male intruder, MIA males emit fewer vocalizations (A) and also produce significantly fewer two step frequency syllables (B) than controls. Data for the number of calls are plotted as medians plus 25% and 75% quartiles, and range. Two groups of such data were compared with the Mann–Whitney test. Significant differences are indicated by $p < 0.05$. USV classification as in Fig. 2.

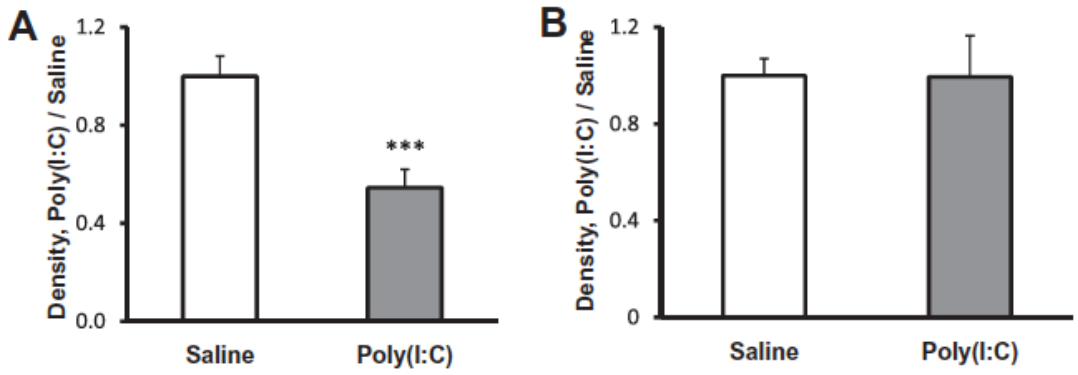

Fig. 4. MIA males display a deficit in female urine-induced scent marking. Compared to controls, MIA males deposit fewer scent marks in the presence of female urine (A). However, no group difference is observed in the scent marking induced by male urine (B). A significant difference is indicated by ***$p < 0.005$.

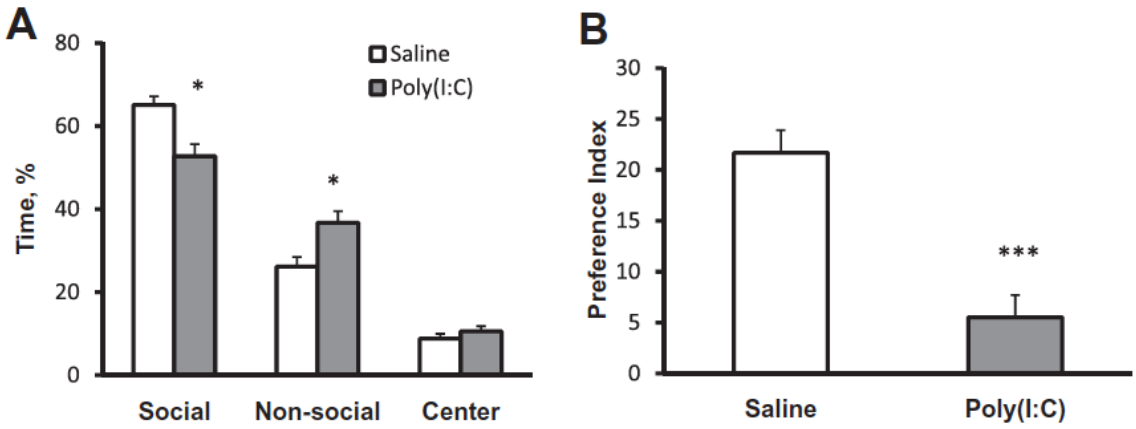

Fig. 5. Adult male offspring from MIA mothers exhibit decreased sociability in the three chamber social test. Compared to controls, MIA males spend significantly less time in the chamber with an unfamiliar mouse and more time in the chamber with an unfamiliar, non-social object (A). The preference index for social object was significantly lower in the MIA offspring (B). Significant differences are indicated by *$p < 0.05$ and ***$p < 0.005$.

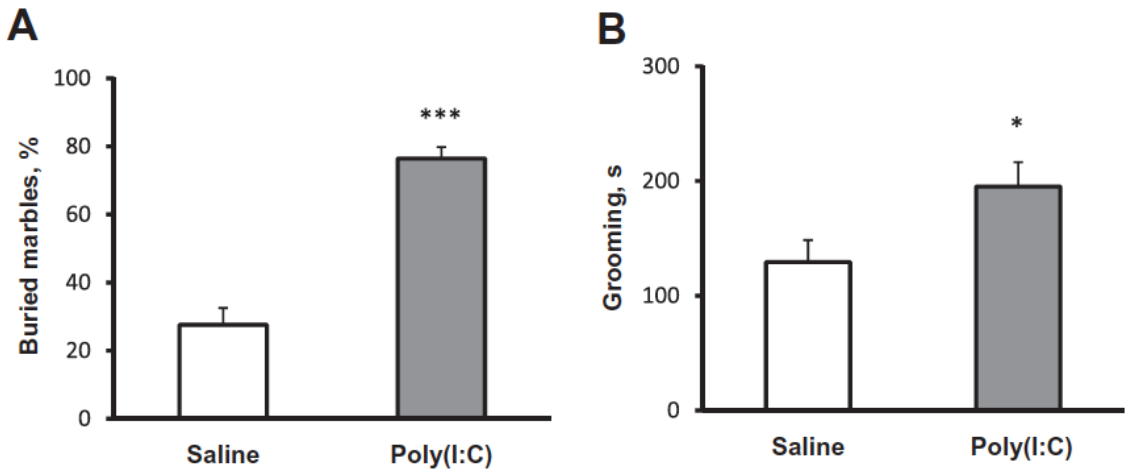

Fig. 6. Offspring of MIA mothers display high levels of repetitive/compulsive behavior. Compared to controls, MIA offspring display extremely high repetitive behavior in the marble burying test (A), and increased self-grooming (B). Significant differences are indicated by *$p < 0.05$ and ***$p < 0.001$.

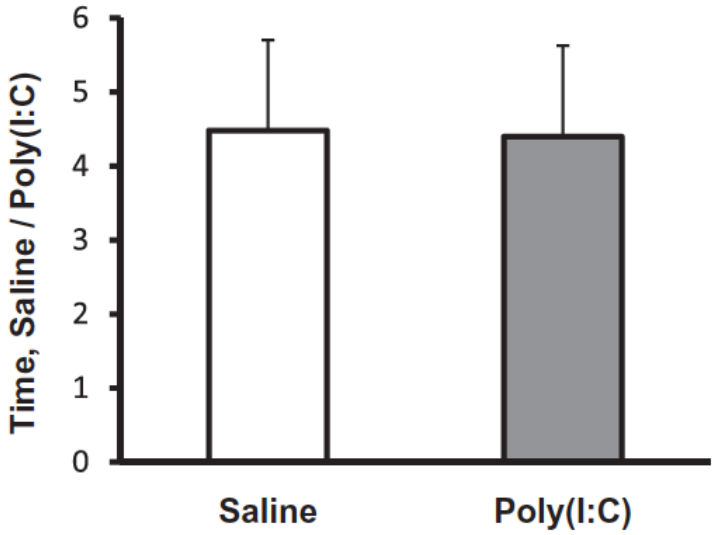

Fig. 7. MIA offspring do not display an obvious olfactory deficit. The data are presented as the ratio of the time spent exploring a scented paper to the time spent exploring a plain water spotted paper.

Table 1
Duration of USV syllables of MIA and control offspring in three social encounters.

	1F	2F	3+F	HM	CV	CX	FT	UP	DN	ST	RC
P10 pup											
Saline	41.1 ± 3.3	49.5 ± 4.5	68.3 ± 7.8	59.5 ± 4.1	22.9±.31	49.2 ± 1.9	18.5 ± 2.0	11.0 ± 0.5	26.8 ± 2.5	3.8 ± 0.7	29.9 ± 2.1
Poly(I:C)	37.0 ± 2.9	48.9 ± 3.5	71.5 ± 6.8	53.4 ± 4.5	20.2 ± 1.5	40.6 ± 1.2*	16.1 ± 1.5	13.9 ± 1.3*	19.7 ± 2.3*	4.2 ± 0.7	23.0 ± 3.0
Male–female											
Saline	58.8 ± 3.9	74.4 ± 4.0	110.8 ± 6.6	97.4 ± 5.1	43.0 ± 2.1	81.5 ± 3.7	24.0 ± 2.7	20.7 ± 1.0	36.8 ± 2.5	4.1 ± 0.3	33.5 ± 2.1
Poly(I:C)	52.9 ± 4.1	66.3 ± 4.8	92.9 ± 6.9*	97.8 ± 9.2	39.0 ± 2.4	68.7 ± 4.8	25.0 ± 2.4	21.9 ± 2.1	29.5 ± 3.3	5.7 ± 1.7	32.7 ± 2.8
Male–male											
Saline	37.4 ± 2.5	47.0 ± 3.0	97.3 ± 11.4	73.1 ± 0.7	22.3 ± 1.2	38.9 ± 3.3	10.6 ± 1.1	15.2 ± 0.7	23.1 ± 2.0	5.2 ± 1.2	19.4 ± 1.9
Poly(I:C)	33.9 ± 4.4	39.0 ± 0.4	82.3 ± 8.7	76.3 ± 8.3	21.8 ± 0.2	34.3 ± 3.3	12.1 ± 0.9	13.5 ± 1.0	17.3 ± 1.3	4.2 ± 0.8	19.6 ± 1.8

Duration for each syllable type is measured in milliseconds. Data are mean values ± SEM. USV classification: 1F, one frequency step; 2F, two frequency step; 3F, three and more frequency step; HM, harmonic; CV, chevron; CX, complex; FT, flat; UP, upward; DN, downward; ST, short.
* $p < 0.05$.

Table 2
Dominant frequency of USV syllables of MIA and control offspring in three social encounters.

	1F	2F	3+F	HM	CV	CX	FT	UP	DN	ST	RC
P10 pup											
Saline	71.1 ± 1.8	70.2 ± 2.2	59.8 ± 1.9	66.8 ± 1.6	84.5 ± 0.8	85.5 ± 1.6	70.8 ± 2.6	86.5 ± 2.1	80.5 ± 1.4	86.0 ± 1.4	78.2 ± 3.0
Poly(I:C)	72.3 ± 2.6	68.6 ± 1.5	59.3 ± 1.9	66.8 ± 1.7	85.8 ± 0.8	86.4 ± 0.7	76.3 ± 2.9	90.4 ± 1.1	82.1 ± 2.0	87.5 ± 1.3	81.9 ± 2.8
Male–female											
Saline	72.0 ± 0.7	71.7 ± 0.6	65.4 ± 3.2	63.6 ± 3.2	77.4 ± 1.2	72.4 ± 1.1	70.5 ± 4.4	7.8 ± 1.4	70.0 ± 3.7	78.6 ± 1.8	73.9 ± 1.1
Poly(I:C)	72.9 ± 1.2	73.8 ± 1.5	69.5 ± 1.2	62.2 ± 3.5	78.5 ± 1.2	72.9 ± 1.0	68.1 ± 3.7	79.0 ± 1.2	75.7 ± 1.5	78.2 ± 1.8	73.1 ± 1.0
Male–male											
Saline	79.6 ± 0.8	81.7 ± 1.2	75.8 ± 2.3	77.3 ± 1.3	86.1 ± 2.1	80.0 ± 1.7	84.4 ± 3.6	82.4 ± 0.6	83.9 ± 1.6	87.1 ± 1.5	80.3 ± 0.9
Poly(I:C)	75.6 ± 2.1	81.3 ± 2.6	69.7 ± 1.1	68.6 ± 1.6	89.2 ± 3.0	80.3 ± 2.3	73.4 ± 3.7*	82.3 ± 1.7	78.8 ± 2.3	82.2 ± 2.1	73.6 ± 2.1

Dominant frequency for each syllable type (at peak of frequency) is measured in kHz. Data are mean values ± SEM. USV classification: 1F, one frequency step; 2F, two frequency step; 3F, three and more frequency step; HM, harmonic; CV, chevron; CX, complex; FT, flat; UP, upward; DN, downward; ST, short.

* $p < 0.05$.

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Chapter 5

Placental regulation of maternal-fetal interactions and brain development

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Abstract

A variety prenatal insults are associated with the incidence of neurodevelopmental disorders such as schizophrenia, autism and cerebral palsy. While the precise mechanisms underlying how transient gestational challenges can lead to later life dysfunctions are largely unknown, the placenta is likely to play a key role. The literal interface between maternal and fetal cells resides in the placenta, and disruptions to the maternal or intrauterine environment are necessarily conveyed to the developing embryo via the placenta. Placental cells bear the responsibility of promoting maternal tolerance of the semi-allogeneic fetus and regulating selective permeability of nutrients, gases and antibodies, while still providing physiological protection of the embryo from adversity. The placenta's critical role in modulating immune protection and the availability of nutrients and endocrine factors to the offspring implicates its involvement in autoimmunity, growth restriction and hypoxia, all factors associated with the development of neurological complications. In this review, we summarize primary maternal-fetal interactions that occur in the placenta and describe pathways by which maternal insults can impair these processes and disrupt fetal brain development. We also review emerging evidence for placental dysfunction in the prenatal programming of neurodevelopmental disorders.

Introduction

A key advance in modern neurobiology is the understanding that the nervous system exhibits lifelong reciprocal interactions with the immune and endocrine systems. These interactions and their high sensitivity to environmental cues means that alterations in one domain leads to changes in another. This is evident in systemic infection, where activation of an immune response in the periphery leads to activation of vagal afferents and their projection areas in the brain, causing changes known as sickness behavior (Dantzer et al., 2008). In the other direction, during the stress response, activation of hypothalamic neurons stimulates the hypothalamic-pituitary-adrenal (HPA) axis and corticosterone production (Dedovic et al., 2009). These effects of stress lead to altered peripheral immune function (Segerstrom and Miller, 2004).

Such plasticity is particularly important during embryonic development, enabling an organism to adapt to the demands of the environment in which it develops and will eventually inhabit. While this ability to reorganize regulatory systems in response to immediate environmental pressures confers an obvious adaptive advantage, it also entails a risk for adverse, long-term effects on physiological functions, particularly if the prenatal environment is discordant with the postnatal environment. That is, priming of fetal development *in utero* may lead the offspring to respond inappropriately to a postnatal environment that is different from that for which it was prepared. This is exemplified in the effect of maternal undernutrition on offspring metabolism and subsequent susceptibility to obesity (Krechowec et al., 2006). Such findings support the concepts of “fetal programming” and the “developmental origins of health and disease,”

which describe how environmental influences on early development initiate molecular responses that impact long-term predisposition for disease.

A variety of maternal and intrauterine insults are known to affect fetal neurodevelopment, but the mechanisms underlying how such transient prenatal challenges can lead to persistent postnatal dysregulation are largely unknown. It is likely that the placenta, as a key regulator of maternal-fetal interactions, plays an important role. Indeed, changes in placental shape and weight are associated with the development of diseases such as hypertension, coronary heart disease and stroke in later life (Barker et al., 1990; Warning et al., 2011). Moreover, the placenta's central role in regulating nutrient transport, endocrine function and immune tolerance implicates its involvement in growth restriction, hypoxia and related neurological complications (Jansson and Powell, 2007; Bale et al., 2010; Fernandez-Twinn and Ozanne, 2010). In this review, we briefly describe some of the complex, maternal-fetal interactions that occur in the placenta. We further discuss the pathways by which maternal perturbations known to alter neurodevelopment also disrupt placental physiology. We place particular focus on murine studies, from which several mechanistic insights into how placental disruptions influence offspring development have been drawn. In closing, we review emerging evidence for a placental role in prenatal programming of neurodevelopmental disorders.

Maternal-fetal interactions in the placenta

Far from being a passive organ, the placenta plays a critical role in orchestrating the sequence and intensity of a series of complex maternal-fetal interactions. In essence, the placenta is of dual origin, comprised of both fetallyand maternally-derived cells (Fig.

1). The decidua, often referred to as the maternal compartment, forms the most superficial layer surrounding the placenta and is densely packed with maternal immune cells. Beneath this, a layer of fetally-derived trophoblast cells secretes hormones and endocrine factors that support both maternal and fetal health. Finally, maternal blood, descending from decidual spiral arteries, and fetal blood, rising through the umbilical arteries, converge in the villous spaces of what is known as the labyrinth layer, in mice, or the chorionic villi, in humans. Here, maternal and fetal blood flow countercurrently and are separated by two layers of fetal trophoblast cells, the syncytiotrophoblasts and the so-called mononuclear trophoblasts, in mice, or villous cytotrophoblasts, in humans. These trophoblasts are critical for regulating the selective entrance of nutrients and oxygen into the fetal bloodstream.

Immune tolerance

The literal maternal-fetal interface resides at the decidual-trophoblast junction of the placenta and across the syncytiotrophoblast cells that form the boundary between maternal and fetal blood spaces in the villous layer (Fig. 1). It was recognized early on that the close contact between maternal and fetal cells at this interface represents an immunological paradox (Medawar, 1953); that is, how can trophoblast cells, which express paternal alloantigens, live harmoniously with maternal leukocytes that are developmentally educated to react against non-self antigens? Of particular concern is the reactivity of a unique population of uterine natural killer (uNK) cells, which constitutes approximately 70% of all decidual leukocytes (Bulmer et al., 2010) and displays cytotoxicity *in vitro*. Maternal macrophages, which form about 20% of all decidual

leukocytes, along with maternal T cells and dendritic cells, retain their effector functions and reside closely with fetal trophoblasts (Scaife et al., 2003). Furthermore, that Rhesus disease involves maternal antibody production against the offspring's paternally-inherited Rh factor demonstrates that the maternal adaptive immune system is capable of reacting against fetal antigens.

On the other hand, anti-fetal immune responses are specifically suppressed during pregnancy (Aluvihare et al., 2004). Remarkably, pregnant mice will accept an allogeneic tumor graft if harboring offspring with matching alloantigens (Tafuri et al., 1995). Furthermore, acceptance of the allograft is limited to the gestational period; after birthing, allografts are rejected. This indicates that the maternal immune system is specifically tolerized to fetal alloantigens during pregnancy.

There are several mechanisms underlying immune tolerance in the placenta, and many of these depend on crosstalk between maternal and fetal cells (Table 1). Paracrine signaling between fetal and maternal cells is critical for establishing a state of immunosuppression. Trophoblast cells secrete a variety of immunosuppressive factors and harbor surface ligands to control immune reactivity through cell-cell interactions. Shedding of trophoblast antigens and trafficking of fetal cells into the maternal circulation are believed to promote classical immune tolerance during major histocompatibility complex (MHC) restriction and maturation of maternal T cells. Of principal importance is that trophoblasts exhibit limited expression of surface MHC alloantigens as a strategic way to evade maternal immune surveillance. Similar mechanisms of host immune evasion are used by tumor cells and the human immunodeficiency virus (HIV) (Collins and Baltimore, 1999; Fruh et al., 1999).

Breaching immunological tolerance at the maternal-fetal interface can result in a number of obstetric complications (Trowsdale and Betz, 2006; Warning et al., 2011). Insufficient immunological tolerance is believed to underlie many cases of pre-eclampsia and miscarriage, which is consistent with epidemiological associations of these conditions with pre-existing autoimmune disease in the mother (Wolfberg et al., 2004; Tincani et al., 2008). A signature of altered immunological status in the placenta is infiltration of uterine and decidual leukocytes into fetal compartments. Lack of tolerance also entails maternal reactivity against paternal antigens on fetal cells, which can result in cytotoxicity, placental necrosis and maternal production of anti-fetal antibodies. Notably, maternal autoantibody production against fetal antigens is involved in a number of developmental disorders, including autism (Braunschweig et al., 2008). Thus, while numerous strategic mechanisms have evolved to accommodate the coexistence of maternal and fetal cells in the placenta, insults which impact placental signaling factors and immune status can lead to deficits in immunological tolerance at the maternal-fetal interface.

Trophoblast invasion

At the same time that maternal and fetal cells in the placenta must interact to maintain immunological tolerance, they also need to coordinate to deliver progressively more nutrients to the growing embryo. This is achieved specifically in hemachorial placentas (where chorionic cells come into direct contact with maternal blood) by specialized groups of trophoblast cells that travel into spiral arteries and initiate vascular remodeling (Moffett and Loke, 2006; Cartwright et al., 2010). Interstitial trophoblasts,

which derive from villous trophoblasts, invade the decidua and spiral arteries. There, they initiate programmed cell death of existing maternal endothelial and smooth muscle cells, opening up blood flow to accommodate the needs of the developing fetus. This process effectively increases the diameter of the spiral arteries, allowing maternal blood to fill the villous spaces at an elevated rate with decreased resistance. Thus, greater levels of nutrients, growth factors and oxygen are transferred to the fetal circulation to promote healthy embryonic development.

Successful trophoblast invasion is mediated by crosstalk between the interstitial trophoblasts and the diverse cell types that they encounter on the journey toward the decidua (Table 2). These interactions result in an intricate sequence of temporallyand spatially-restricted changes in gene expression. For example, interactions with distinct placental cell types trigger invading trophoblast cells to change their display of surface adhesion molecules as they migrate up to the decidua, progressively acquiring characteristics of endothelial cells. Furthermore, invading trophoblasts upregulate expression of molecules that help to digest the extracellular matrix barrier. The extent of invasion is further governed by cytokine signaling between decidual cells and interstitial trophoblasts. Direct cell-cell interactions between trophoblasts and decidual leukocytes may also regulate invasion and spiral artery modification.

Modification of spiral arteries/vascular remodeling

The transformation of spiral arteries after trophoblast invasion reflects a culmination of several molecular events that effectively alter the vascular properties of the spiral arteries. The invasive trophoblasts themselves are important for inducing

apoptosis of endothelial cells and stromal cells in the vasculature. Even prior to trophoblast invasion, decidual immune cells localized near the vessel walls initiate early arterial vacuolization and dilation (Hazan et al., 2010). Moreover, it is believed that the phagocytic properties uNK cells aid in the clearance of apoptosed endothelial and stromal cells surrounding the spiral arteries, making way for new endothelial-like trophoblasts to reconstitute the vasculature.

Both trophoblast invasion and vascular remodeling are intimately tied to fetal growth and development. Disruption of either process leads to altered maternal blood flow into the villous spaces and inappropriate exchange of nutrients and respiratory gases. This can be detected histologically by shallow extravillous trophoblast invasion or over-infiltration of the decidual matrix and suboptimal vascularization. Associated changes in blood circulation and flow resistance can be conveyed by altered experimental parameters during placental perfusion. These molecular disruptions can form the mechanistic bases for fetal malnutrition, intrauterine hypoxia and altered placental weight or birth weight (Roberts et al., 2001; Redmer et al., 2004; Sibley et al., 2005; Cartwright et al., 2007). A variety of medical conditions arise from such complications, including premature birth, pre-eclampsia and intrauterine growth restriction (IUGR) (Sibley et al., 2005; Cudihy and Lee, 2009), and these conditions are further associated with a number of neurodevelopmental disorders, such as cerebral palsy and schizophrenia (Preti et al., 2000; Jarvis et al., 2006; Redline, 2006; Clarke et al., 2011; O'Callaghan et al., 2011).

Prenatal effects on the placenta and neurodevelopment

It is clear that the molecular mechanisms regulating normal placental functions are tightly intertwined, governed by both cells at the maternal-fetal interface and soluble factors in the local microenvironment. Signaling of cytokines, growth factors and hormones are central to the cross-talk between maternal and fetal cells in the placenta, dictating the gene expression changes that modulate their physiological functions. Also, the activation states of decidual immune cells can influence not only immunological tolerance at the maternal-fetal interface, but also the production of soluble factors and types of cell-cell interactions that influence trophoblast invasion and vascular remodeling. Overall, the cellular interactions underlying these processes are critical for establishing normal placental architecture, delivering sufficient oxygen and nutrients to the fetus and protecting the fetus from maternal reactivity. Thus, a proper intrauterine environment is fundamental to the success of the placenta and pregnancy in supporting healthy fetal development.

Maternal insults that disrupt the fine interplay of signaling networks at the maternal-fetal interface can alter placental capacity and complicate fetal development and behavior. Given that maternal challenges are conveyed to the fetus via the placenta, many maternal insults lead to altered intrauterine environments and consequent placental pathology. Maternal anemia, for example, is associated with altered placental angiogenesis, intrauterine hypoxia and perinatal brain injury in the offspring (Kadyrov et al., 1998; Fowden et al., 2008). Here we discuss maternal immune activation (MIA) as a major factor known to disturb placental physiology and lead to alterations in offspring brain development with long-term consequences.

Maternal immune activation (MIA)

While normal pregnancy involves several mechanisms to promote immunosuppression and immune evasion at the maternal-fetal interface, the placenta retains the ability to detect and respond to infection and inflammation. Placental cells express a variety of pattern recognition receptors (PRRs) that recognize unique microbe-associated molecular patterns (MAMPs) expressed extracellularly or intracellularly on microorganisms. PRRs such as mannose receptors, NOD-like receptors and Toll-like receptors (TLRs) are expressed not only on placental immune cells, but also on trophoblasts themselves (Koga and Mor, 2010). In fact, all 10 TLRs, in addition to many related co-receptors and accessory proteins, are found in the human placenta, rendering the placenta responsive to MAMPs on bacteria, viruses, parasites and fungi.

In animal models, respiratory infection or injection of MAMPs, such as the bacterial cell wall component lipopolysaccharide (LPS) or the synthetic double-stranded RNA poly(I:C) (to mimic viral infection), into pregnant dams triggers a maternal inflammatory response that can lead to placental pathology and subsequent harm to the fetus (Koga and Mor, 2010). The precise developmental effects of MIA depend on the specific antigen used, as well as the dosage, route and timing of administration. Early gestational injection of LPS or poly(I:C), for example, can lead to implantation failure or fetal resorption, while exposure during late gestation can induce pre-term birth (Ilievski et al., 2007). Relatively low-dose LPS or poly(I:C) injection into rodents during mid-to-late-gestation yields offspring with schizophreniaand autism-related developmental abnormalities in the absence of overt obstetric complications (Patterson, 2011). Common

to these variations of MIA, however, is a consistent upregulation of pro-inflammatory cytokines, chemokines and reactive oxygen species in the maternal blood and placenta.

MIA induces the production of soluble pro-inflammatory factors that have access to placental cells via maternal blood in the spiral arteries and intervillous spaces. Moreover, MIA can alter the immune status of decidual leukocytes, including upregulation of activation markers and increased production of pro-inflammatory cytokines by uNK cells, macrophages and granulocytes (Zhang et al., 2007; Renaud and Graham, 2008; Hsiao and Patterson, 2011). Not surprisingly, these effects on uterine immune cells can lead to further disruption of immunological tolerance, cytoarchitecture and blood circulation in the placenta. Maternal LPS injection, for instance, induces necrosis and edema of the labyrinth layer, infiltration of maternal immune cells into fetal placental tissues and decreased placental perfusion (Girard et al., 2010; Carpentier et al., 2011). LPS-mediated activation of TLR4 signal transduction also inhibits the migration of trophoblast cells (Abrahams et al., 2005), which may form the basis of observed decreases in placental circulation.

In response to maternal poly(I:C) injection, however, the placenta experiences a dramatic upregulation of pro-inflammatory cytokines and activation of decidual immune cells, in the absence of obvious pathophysiology (Zhang et al., 2007; Hsiao and Patterson, 2011). The maternal pro-inflammatory response is further relayed to fetal spongiotrophoblast and labyrinth cells, which activate JAK/STAT signaling pathways and alter expression of several genes, including those that encode important endocrine factors such as growth hormone and insulin-like growth factor 1 (IGF1) (Fatemi et al., 2011)**.** Importantly, these effects on the placenta, as well as MIA-induced behavioral

abnormalities in the offspring, are dependent on IL-6 signaling (Smith et al., 2007; Hsiao and Patterson, 2011). In addition to these changes in factors that influence fetal health, some pro-inflammatory cytokines, such as IL-6 and IL-1β, can cross the placenta and enter the fetal bloodstream (Dahlgren et al., 2006; Girard et al., 2010). Thus, soluble factors produced in response to maternal insults can activate cells at the maternal-fetal interface and potentially be passively transported directly into the fetal circulation. These findings highlight the key role of the placenta in amplifying the maternal inflammatory response and potentiating detrimental effects on embryonic health. They further contribute to a growing number of MIA studies that demonstrate a critical role of cytokine signaling in triggering molecular cascades that ultimately impair fetal development.

The long-term effects of MIA on the offspring include a variety of behavioral, histological and immunological abnormalities that relate to symptoms of human autism and schizophrenia. Adult MIA offspring exhibit deficits in pre-pulse inhibition, latent inhibition, open field exploration, social interaction and vocal and olfactory communication, as well as increased anxiety and repetitive/stereotyped behavior (Malkova et al., 2012). They also exhibit enlarged ventricles, a hallmark neuropathology of schizophrenia and a spatially-restricted deficit in cerebellar Purkinje cells, a characteristic neuropathology of autism, among several other neuropatholgies (Patterson, 2007). Young and adult MIA offspring also display autism-related immune dysregulation, including decreased T regulatory cells and hyper-responsive CD4+ T cells (Hsiao et al., 2011). Together, these effects of MIA in animal models support large epidemiological studies linking maternal viral, bacterial and parasitic infection to

increased risk of schizophrenia or autism in the offspring (Patterson, 2007). Also supporting this body of work are associations between these mental illnesses and elevated cytokines in amniotic fluid and maternal serum.

The placenta in programming of neurodevelopmental disorders

Altered placental function and the release of deleterious factors to the fetus, in response to such challenges as MIA, are important risk factors for the pathogenesis of neurodevelopmental disorders. Several maternal insults, including maternal infection and maternal malnutrition, increase susceptibility to IUGR, and all three factors are epidemiologically linked to schizophrenia, autism and cerebral palsy in the offspring (Brown and Susser, 2008; Atladottir et al., 2010; Brown and Patterson, 2011; O'Callaghan et al., 2011). Placental pathologies relating to vascular impairments, including chorionic vessel thrombi, villous edemas and vascular necrosis, are prevalent in cerebral palsy (Redline, 2006). In addition, obstetric complications are associated with increased risk for schizophrenia and can predict treatment responses in schizophrenic individuals (Alvir et al., 1999; Preti et al., 2000). Exposure to obstetric complications and immune dysregulation are similarly linked to autism susceptibility in the offspring (Limperopoulos et al., 2008; Gardener et al., 2009; Sacco et al., 2010). In one study, trophoblast inclusions were increased in placental tissue derived from births of individuals who developed autism spectrum disorder (Anderson et al., 2007). In addition, chorioamnionitis, or inflammation of the fetal placental membranes, is associated with impairments in social interaction and communication in autistic children (Limperopoulos et al., 2008).

That placental pathologies are associated with brain injury and altered behavior suggests that dysfunction at the maternal-fetal interface can contribute to the pathogenesis of neurodevelopmental disorders (Fig. 2). Whether placental impairments can directly cause the disorders in subsets of individuals seems likely but remains to be firmly established. Studies of animal models of intrauterine infection and IUGR, where gestational challenges are confined to the uteroplacental compartment, demonstrate that primary insults to the placenta can manifest in perinatal brain damage in the offspring. Rodents, ewes and rabbits subjected to such placental challenges exhibit a variety of neuropathologies, including altered astrocyte development, microglial activation, white-matter damage and impaired blood brain barrier integrity (Hutton et al., 2008; Bassan et al., 2010). Notably, uteroplacental inflammation is sufficient to induce the expression of the apoptotic markers, caspase-3 and 4-hydroxynonenal, by Purkinje cells of the fetal ovine cerebellum (Hutton et al., 2007). This is reminiscent of the Purkinje cell loss characteristic of autistic brains and observed in other neurodevelopmental disorders such as schizophrenia. It will be interesting to further assess the downstream effects of primary insults to the placenta on offspring behavioral development.

Furthermore, the placenta is known to regulate the synthesis of neuroactive factors throughout gestation that could influence fetal brain development (Petraglia et al., 2010). Recent findings in mice demonstrate that the placenta serves as a major source of serotonin to the fetal forebrain (Bonnin et al., 2011). Delivery of the precursor tryptophan through the maternal uterine artery leads to accumulation of newly synthesized serotonin in the placenta and fetal circulation, demonstrating the ability of the placenta to synthesize serotonin and transport it to the fetus. In contrast, delivery of a tryptophan

hydroxylase antagonist to the placenta sufficiently inhibits placental serotonin synthesis and reduces levels of forebrain serotonin in corresponding embryos. These findings contribute to a growing number of studies that illuminate the key role of the placenta in *de novo* synthesis of neuroactive factors that are necessary for normal brain development (Hirst et al., 2009; Petraglia et al., 2010). Interestingly, placental infection and inflammation is associated with disruptions to the kynurenine/tryptophan pathway in the placenta, which may have corresponding effects on serotonin production and neural development.

The placenta is also a primary hematopoietic stem cell (HSC) niche during pregnancy, harboring a large population of HSCs during midgestation that are believed to seed the fetal liver (Gekas et al., 2005). Importantly, HSC development occurs in the placental vasculature independently of blood flow, supporting the finding that the placenta itself produces definitive hematopoietic cells that encompass both myeloerythroid and lymphoid potential (Rhodes et al., 2008). Thus, prenatal insults that influence placental physiology may also impact placental HSC development and postnatal immunity. Indeed, development of the immune system and responses of effecter immune cells are influenced by early life environments and changes in microenvironmental cues such as cytokines (Sobrian et al., 1997; Coe and Lubach, 2000). In addition, altered immunity is frequently associated with neurodevelopmental disorders, such as schizophrenia and autism spectrum disorders (Patterson, 2009; Onore et al., 2011). It will be important to assess whether levels or properties of placental HSCs are altered by such prenatal insults as intrauterine infection.

Additional multi-disciplinary studies are needed to elucidate the mechanisms by which the placenta guides normal fetal brain development and to gain insight into its role in the developmental programming of long-term health and disease. Specifically, additional models that involve primary insults to the placenta itself, rather than secondary effects on the placenta resulting from primary maternal challenge, will help focus research on placenta-specific effects on fetal development in the absence of confounding maternal factors. Lentivirus-mediated, placenta-specific transgenesis (Okada et al., 2007) and the utilization of transgenic mice harboring placenta-specific drivers such as trophoblast-specific protein alpha (*Tpbpa*) and placental lactogens (*Pl1* and *Pl2*) will facilitate studies on the effects of targeted placental manipulations on offspring development. It will be particularly important to determine the effects on disease-relevant behaviors and neuropathology. Finally, the study of placental responses to maternal or intrauterine insults offers the potential to identify early targets for prevention of later-life disease. Such promise can be seen with the increasing use of prenatal magnesium sulfate for promoting fetal neuroprotection and preventing cerebral palsy and substantial motor dysfunction in at-risk infants (Doyle, 2012). While the precise basis for this protection is unclear, several studies demonstrate anti-inflammatory, anti-apoptotic and vasodilatory properties of magnesium sulfate in the placenta (Kovac et al., 2003; Holcberg et al., 2004; Dowling et al., 2012). Further studies into the role of the placenta in regulating fetal development will shed light on how alterations in a variety of interactions at maternal-fetal interface may form the basis of early life programming of neurological, metabolic, as well as immunological disorders.

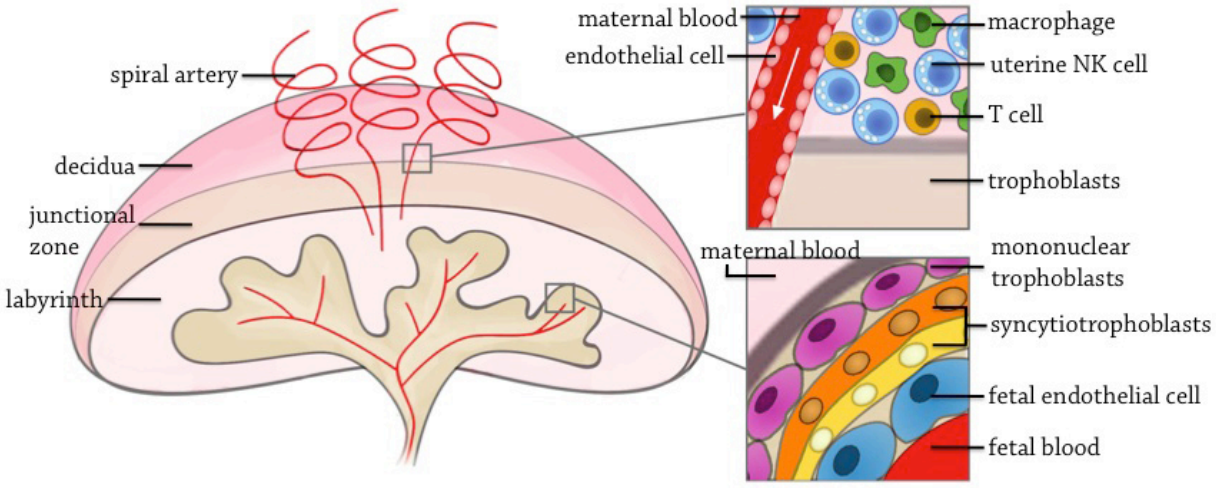

Figure 1. The maternal-fetal interface in the murine placenta. The direct interaction between maternal and fetal cells during gestation occurs in the placenta. The placenta is of dual origin, with the outer decidual layer composed almost entirely of maternal immune cells, while the underlying junctional zone and labyrinth layers (chorionic villous layer, in human) are comprised exclusively of fetally-derived trophoblasts and leukocytes. Maternal immune cells and endothelial cells of the spiral arteries are juxtaposed along trophoblasts at the boundary between the decidua and junctional zone (upper right). In the labyrinth layer, intervillous spaces are lined by fetal syncytiotrophoblast cells, mononuclear trophoblast cells and fetal endothelial cells that separate maternal from fetal blood (lower right).

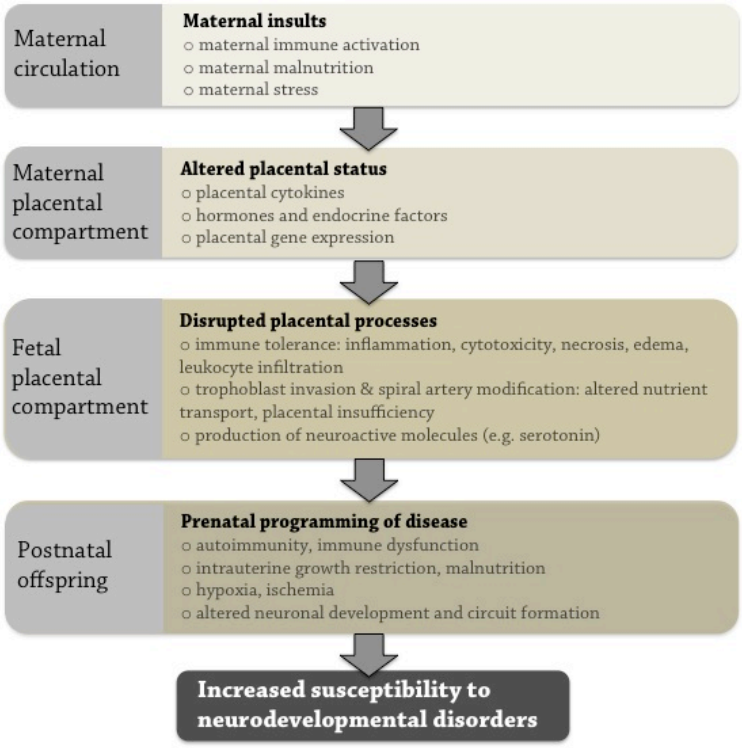

Figure 2. A placental role in developmental programming. Maternal and intrauterine challenges are necessarily conveyed to the developing offspring via the placenta. Maternal insults such as immune activation, malnutrition and stress result in altered composition of the maternal blood, which descends through the spiral arteries to fill the placental blood spaces. Changes in placental cytokines and endocrine factors can alter placental gene expression at the maternal-fetal interface. In light of the critical role of signaling factors in regulating normal placental processes, these alterations can further disrupt the maintenance of immune tolerance, progression of trophoblast invasion and spiral artery modification, or the *de novo* production of neuroactive molecules in the placenta. Placental pathophysiology contributes to a variety of obstetric complications and impairments to fetal development, such as hypoxia, growth restriction or immune dysregulation. These challenges further elaborate detrimental effects on early brain development, increasing susceptibility to neurodevelopmental disorders.

Table 1 Immunological Tolerance at the Maternal-Fetal Interface: Molecular Mechanisms Initiated by Trophoblast Cells

Mechanism		Function
Immuno-suppression via paracrine signaling	IDO secretion	Inhibit T cell responses via tryptophan depletion
	VIP secretion	Promote differentiation of regulatory T cells
	Soluble HLA-G secretion	Protect against NK-cell mediated cytotoxicity
Cell-cell interaction	FasL expression	Induce apoptosis upon ligation of Fas expressed on activated leukocytes
Induction of maternal tolerance	Trophoblast shedding	Expose the maternal circulation to paternal antigens shed from trophoblast cells
	Fetal microchimerism	Expose the maternal circulation to paternal antigens via direct trafficking of fetal cells
Immune evasion	Lack of surface MHCII	Elude recognition by CD4+ helper T cells
	Lack of HLA-A and HLA-B	Elude recognition by CD8+ cytotoxic T cells
	Expression of HLA-C, HLA-E and HLA-G	Draws decreased recognition by the immune system because these MHC isotypes exhibit low to undetectable levels of polymorphism

Table 2 Molecular Mechanisms Underlying Trophoblast Spiral Artery Modification at the Maternal-Fetal Interaction

Mechanism		Function
Altered gene expression	PECAM-1 and VE-cadherin expression	Alter display of surface adhesion molecules to aid in the migration towards the decidua and to establish characteristics of endothelial cells
	Matrix metalloproteinase expression	Digest the extracellular matrix during the travel toward the myometrium
Paracrine signaling to decidual cells	HGF, EGF and LIF secretion	Factors to stimulate invation
	TGFb, IFNy and IL-11 secretion	Factors to limit the extent of invasion
	TNFa and TRAIL secretion	Initiate caspase activation and apoptosis of endothelial cells and stromal cells
Cell-cell interactions	Surface HLA-C and HLA-G expression	Bind to killer immunoglobulin receptors on uNK cells or LIL receptors on decidual macrophages to regulate cytokine expression

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Chapter 6

Activation of the maternal immune system induces endocrine changes in the placenta via IL-6

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Abstract

Activation of the maternal immune system in rodent models sets in motion a cascade of molecular pathways that ultimately result in autismand schizophrenia-related behaviors in offspring. The finding that interleukin-6 (IL-6) is a crucial mediator of these effects led us to examine the mechanism by which this cytokine influences fetal development *in vivo*. Here we focus on the placenta as the site of direct interaction between mother and fetus and as a principal modulator of fetal development. We find that maternal immune activation (MIA) with a viral mimic, synthetic double-stranded RNA (poly(I:C)), increases IL-6 mRNA as well as maternally-derived IL-6 protein in the placenta. Placentas from MIA mothers exhibit increases in CD69+ decidual macrophages, granulocytes and uterine NK cells, indicating elevated early immune activation. Maternally-derived IL-6 mediates activation of the JAK/STAT3 pathway specifically in the spongiotrophoblast layer of the placenta, which results in expression of acute phase genes. Importantly, this parallels an IL-6-dependent disruption of the growth hormone-insulin-like growth factor (GH-IGF) axis that is characterized by decreased GH, IGFI and IGFBP3 levels. In addition, we observe an IL-6-dependent induction in pro-lactin-like protein-K (PLP-K) expression as well as MIA-related alterations in other placental endocrine factors**.** Together, these IL-6-mediated effects of MIA on the placenta represent an indirect mechanism by which MIA can alter fetal development.

Introduction

Both autism and schizophrenia are relatively common disorders with often tragic, lifelong consequences. Several susceptibility genes and environmental agents have been identified as risk factors, but few cases of autism or schizophrenia can be traced to a known cause. Maternal infection is regarded as a principal, non-genetic cause of schizophrenia, and is also associated with increased risk for autism in the offspring (reviewed by Brown & Derkits, 2010; Patterson, 2009; Atladóttir et al., 2010). In a mouse model of this risk factor, the offspring of mice injected with poly(I:C) dsRNA as a viral mimic develop behaviors and brain pathology consistent with those seen in human autism and schizophrenia (Lee et al., 2007; Makinodan et al., 2008; Meyer et al., 2006, 2008; Nyffeler et al., 2006; Shi et al., 2003, 2009; Smith et al., 2007; Ito et al., 2010; Ozawa et al., 2006; Piontkewitz et al., 2009; Li et al., 2009; Winter et al., 2009; Zuckerman et al., 2003; Zuckerman & Weiner, 2005). These effects require a key mediator, the cytokine interleukin-6 (IL-6). Maternal injection of IL-6 alone is sufficient to cause the behavioral abnormalities seen in the offspring following maternal poly(I:C) injection or respiratory infection (Samuelsson et al., 2006; Smith et al., 2007). Conversely, co-injection of an antibody that neutralizes endogenous IL-6 along with poly(I:C) completely prevents the prepulse inhibition (PPI), latent inhibition (LI), exploratory and social deficits in the offspring caused by MIA (Smith et al., 2007). Furthermore, poly(I:C) injection of pregnant IL-6 knockout (KO) mice results in no such behavioral deficits. IL-6 is also required for induction of transcriptional changes as co-injection of anti-IL-6 and poly(I:C) normalizes 92% of the MIA-induced changes in gene expression in brains of adult offspring (Smith et al., 2007).

The importance of IL-6 in mediating the development of schizophrenic and autistic endophenotypes in rodents is further supported by post-mortem studies of human subjects. The significant increases in pro-inflammatory cytokines, including IL-6, in fetal brains shortly after MIA in rodents (Meyer et al., 2008) are also observed in the brains of adult schizophrenic and autistic patients (Vargas et al., 2005; Arion et al., 2007; Saetre et al., 2007; Garbett et al., 2008; Li et al., 2009). IL-6 is also elevated in sera from living schizophrenic individuals (Potvin et al., 2008; Maes et al., 2002), and many pro-inflammatory cytokines are increased in the cerebrospinal fluid and sera from living autistic individuals (Pardo et al., 2006; Chez, 2007; Ashwood et al., 2010). Furthermore, an IL-6 receptor polymorphism is associated with schizophrenia (Sun et al., 2008), and increased IL-6 levels are associated with several other environmental risk factors for schizophrenia, including maternal stress, maternal malnutrition and obstetric complications.

Despite demonstration of the critical role of IL-6 in mouse MIA models and in human schizophrenia and autism, the mechanism underlying how IL-6 acts to disrupt early brain development is unknown. IL-6 can act directly on progenitor cells to regulate fetal neurogenesis and gliogenesis (reviewed by Deverman & Patterson, 2009). IL-6 can also act at the maternal-fetal interface to alter many parameters that influence fetal growth, including nutrient transfer, anoxia and vascular permeability (Jones et al., 2009). Moreover, IL-6 can disrupt the immunological balance of the placenta, altering Th1/Th2 homeostasis, activation of uterine immune cells, and maintenance of maternal tolerance (Paul et al., 2003; Zimmerman et al., 2007; Arad et al., 2005; reviewed by Jonakait, 2007).

We have studied the pathway of IL-6 action in the placenta to gain insight into the molecular processes that can lead to the postnatal manifestation of behavioral abnormalities. We confirm that MIA elevates IL-6 protein and mRNA expression in the placenta (Smith et al., 2007; Meyer et al., 2008; Gilmore et al., 2005; Koga et al., 2009). We determine the relative contribution of maternally-derived versus fetally-derived IL-6 to the observed increases in placenta IL-6 levels. We identify activated decidual leukocytes as a likely source of MIA-induced placental IL-6, and we localize the site of maternal, IL-6-dependent STAT3 activation to fetal cells in the placental spongiotrophoblast layer. Finally, we report alterations in maternal-placental hormones and endocrine factors that may have important consequences for fetal development.

Results

Maternal IL-6 exposure yields offspring with behavioral abnormalities similar to those seen in MIA offspring.

In the mouse MIA model, it was previously shown that the cytokine IL-6 is necessary and that a single IL-6 injection is sufficient for the development of behavioral and transcriptional changes in the offspring (Smith et al., 2007). Here we confirm and extend those data. The offspring of pregnant mice injected with 5 mg of rIL-6 on E12.5 display decreased PPI compared to control offspring (Fig. 1A). Schizophrenic (Wynn et al., 2004) and autistic (Perry et al., 2006) individuals also exhibit abnormal PPI. PPI is a measure of sensorimotor gating, attention and distractibility. It refers to the inhibition of a startle response when the startling stimulus (120 db pulse) is preceded by a smaller, non-startling stimulus (5 or 15 db pre-pulse).

LI is a measure of the ability to disregard irrelevant stimuli and refers to the inhibited acquisition of a conditioned stimulus when a subject has been exposed to the stimulus repeatedly prior to pairing with an unconditioned response. The disruption of LI seen in MIA and rIL-6 offspring mimics that which is characteristic of cognitive deficits in schizophrenia (Weiner, 2003). Adult offspring of rIL-6-injected mothers display elevated freezing compared to saline controls in response to the conditioned acoustic cue. This is indicative of decreased LI compared to the non-pre-exposed (NPE) group (Fig. 1B). A similar deficit is observed in poly(I:C) offspring compared to controls.

Offspring of rIL-6-injected mice exhibit decreased exploration of the open field (Fig. 1C). Compared to controls, rIL-6 offspring display fewer center entries, decreased center duration and decreased total distance traveled. Reluctance to enter the center area of an open arena is indicative of heightened anxiety under mildly stressful conditions. The decreased exploration exhibited by MIA and rIL-6 offspring is relevant to schizophrenia and autism-related anxiety-like behavior.

Together, these data indicate that a single exposure to rIL-6 at mid-gestation causes offspring to develop autism and schizophrenia-related behavioral abnormalities similar to those induced by maternal injection of poly(I:C). Previous studies involving the injection of poly(I:C) into IL-6 KO mice, or the co-injection of poly(I:C) with blocking antibody against IL-6, demonstrated that IL-6 is necessary for mediating the effect of MIA on the development of behavioral abnormalities in offspring (Smith et al., 2007). Therefore, in order to elucidate the mechanism by which the maternal immune response affects fetal brain development, we focus on the IL-6 signaling pathway.

The placenta is a site of IL-6 induction in response to MIA.

To explore how an immune challenge that is administered to the mother can lead to changes in the development of offspring, we examined the placenta as the site of direct contact between the maternal and fetal systems. Shortly after poly(I:C) injection, pro-inflammatory cytokines including IL-6 are up-regulated in the maternal circulation (Gilmore et al., 2005; Smith et al., 2007; Meyer et al., 2008; Koga et al., 2009). This signal is transmitted to the placenta, where levels of IL-6 and other pro-inflammatory cytokines are elevated. A striking increase in IL-6 protein is seen in the placenta by 3 hours post-poly(I:C) injection; poly(I:C) placentas exhibit an approximately 17-fold increase over saline injection controls (Fig. 2A). IL-6 levels remain elevated for over 24 hours after MIA, at levels over 4 times greater than in placentas from saline-injected mothers. Moreover, maternal poly(I:C) injection leads to a 16-fold up-regulation of placental IL-6 mRNA expression by 3 hours post-poly(I:C) injection (Fig. 2B). Tumor necrosis factor α (TNFa) and IL-1β expression are up-regulated in poly(I:C) placentas as well, approximately 3-fold over the level in the saline control. A trend for increases is also observed for placental expression of IL-17 and IL-10 in MIA placentas. However, there is no significant difference in expression of leukemia inhibitory factor (LIF), and interferon γ (IFNγ) is not detected in poly(I:C) and saline placentas.

Increased levels of placental IL-6 are maternally-derived.

Elevated IL-6 protein in MIA placentas can come from the mother (e.g. bloodstream, decidual cells, uterine immune cells) and/or the fetus (e.g. bloodstream, trophoblasts, endothelial cells). To explore the relative contribution of the maternal and

fetal pools of IL-6, we crossed IL-6 +/females with IL-6 -/males, and injected pregnant females with either poly(I:C) or saline on E12.5. This creates a system to assay the role of IL-6 production from the fetal compartment: pregnant mothers are able to produce IL-6, whereas approximately one-half of the offspring (and the cells that comprise the spongiotrophoblast and labyrinth) will be null for the IL-6 allele, and thus incapable of generating IL-6 transcript. The remaining heterozygous conceptuses serve as internal controls. We also mated IL-6 -/females with IL-6 +/males to test the necessity of maternal/decidual IL-6 production (and sufficiency of fetal IL-6 production) in mediating the effects of MIA on embryonic development. It is important to note that in this latter paradigm, the decidua (the superficial layer of the placenta) and cells that fill the maternal blood spaces within the spongiotrophoblast and labyrinth, will still be of maternal genotype.

Injection of poly(I:C) into pregnant IL-6 +/females induces the expected MIA response that is characterized by elevated placental IL-6 levels by 3 hours post-injection (Fig. 2C). Note that, compared to WT, IL-6 heterozygous mice display a weaker and more transient MIA response at 3 hours post-injection; placental IL-6 levels are increased ~5-fold over controls, while a ~17-fold induction is seen in WT mice. Also, the placental IL-6 induction lasts between 6 and 24 hours in heterozygous mice, but lasts over 24 hours in WT mice. This indicates that maternal IL-6 gene dosage affects the IL-6 response to MIA in the placenta.

Fetal or trophoblast expression of IL-6 contributes little to the pool of IL-6 protein, as there is no significant difference between IL-6 levels in IL-6 -/placentas from +/mothers compared to IL-6 +/placentas from +/mothers (Fig. 2C). Negligible levels of

IL-6 are detected in IL-6 +/and -/placentas derived from saline or poly(I:C)-injected IL-6 -/mothers (Fig. S1). Thus, the IL-6 protein response to MIA in the placenta is maternally-derived.

MIA activates uterine NK cells, granulocytes and macrophages that may contribute maternal IL-6 in the placenta.

The finding that the placenta exhibits elevated levels of both IL-6 protein and mRNA in response to MIA suggests that placental IL-6 is supplied by not only by the maternal bloodstream, but also by the maternal cells within the placenta. We examined decidual immune cells as candidate sources of maternal IL-6 production. The decidua is the lining of the placenta that contains maternal vasculature and leukocytes, including a distinctive lymphocyte population of uterine NK (uNK) cells. By flow cytometry we assessed immune subtypes in the placenta for expression of CD69, a surface glycoprotein acquired during early lymphoid activation and inducibly expressed by T cells, B cells, NK cells, monocytes, neutrophils and eosinophils.

Compared to saline controls, decidual cell suspensions generated from poly(I:C) injected mothers exhibit elevated CD69 expression in DBA lectin+ uNK cells, CD11b+ macrophages and Gr-1+ granulocytes (Fig. 3A,B). Similarly activated cells are observed in decidual cell suspensions from IL-6 -/mothers injected with poly(I:C), indicating that initial activation of placental immune cells occurs independently of IL-6 action. The increase in CD69+ innate immune cells coincides with an overall increase in placental CD69 expression (Fig. 3C). There is no significant difference in the percentages of these immune subtypes in response to MIA (Fig. S2). Furthermore, there is no apparent

difference in localization of uNK or macrophage cells, as demonstrated by immunofluorescence staining with DBA lectin and F4/80 (data not shown). Because CD4+, CD8+ and B220+ cells represent minor lymphocyte populations in the E12.5 placenta, low levels of these cells are detected in saline and poly(I:C) decidual cell suspensions (Fig. S2). The significant increase in activated maternal leukocytes and lymphocytes in MIA placentas suggests that decidual uNK cells, macrophages and granulocytes are a likely source of maternally-derived IL-6 in response to MIA.

Maternal IL-6 activates the JAK/STAT pathway in the fetal compartment of the placenta.

In order to localize the site(s) of IL-6 action within the placenta, we searched for downstream markers of IL-6 signaling. IL-6 and other IL-6 family cytokines, such as LIF, IL-11 and ciliary neurotrophic factor, signal by binding cytokine-specific membrane receptors to trigger further signal transduction: intracellular JAK proteins dimerize and auto-phosphorylate to activate downstream kinase activity, which ultimately leads to the phosphorylation of STAT transcription factors and global changes in gene expression, including increased transcription of the acute phase genes *SOCS3*, *Pim1*, *TIMP1* and *NOS2*. In particular, IL-6 is known to signal through STAT3 and STAT1. We performed histological staining for phosphorylated (p)STAT3 and pSTAT1 in WT mice injected with poly(I:C) or saline, and also assayed gene expression of downstream markers of IL-6 activity. To evaluate whether changes were initiated specifically by IL-6, we assessed STAT activation and downstream gene expression in IL-6 +/or -/placentas from poly(I:C) or saline-injected IL-6 +/or -/mothers.

Unlike saline controls, STAT3 is phosphorylated in the poly(I:C) spongiotrophoblast layer (Fig. 4) at 3 and 6 hours post-injection. This region of the placenta is comprised of a layer of fetal trophoblast cells that provides both structural support and hormones to maternal and fetal tissues. Widespread pSTAT3 staining is seen in IL-6 +/and -/spongiotrophoblasts from IL-6 +/mothers injected with poly(I:C). This staining is, however, absent in IL-6 +/and -/spongiotrophoblasts from IL-6 -/mothers, further confirming a specific effect of maternally-derived IL-6 on STAT3 activation in the placenta. There is no significant difference in the number of pSTAT3-positive cells in IL-6 +/versus -/spongiotrophoblasts from +/mothers, indicating a negligible effect of fetally-derived IL-6 on STAT3 activation (Fig. S3-A). No pSTAT1 staining is detected in the spongiotrophoblast layer of poly(I:C) or saline placentas (Fig. S3-B). Together, these data indicate that maternal IL-6 plays a specific role in mediating gene expression changes through STAT3 activation in the spongiotrophoblast layer of MIA placentas.

MIA-induced activation of the STAT3 pathway coincides with a strong increase in SOCS3 expression in placentas from poly(I:C)-injected mothers (Fig. 4B). Expression of Pim1, TIMP1 and NOS2 is also increased in MIA placentas. Increased SOCS3 expression is similarly observed in IL-6 +/placentas from poly(I:C)-injected IL-6 +/mothers (Fig. 4C). Note that the up-regulation of SOCS3 expression in IL-6 heterozygous MIA placentas is significantly attenuated compared to that in IL-6 WT placentas. This effect is similarly observed with MIA-induced IL-6 expression, as described above, and provides further evidence that maternal IL-6 gene dosage regulates the intensity and duration of the placental pro-inflammatory response to poly(I:C). Moreover, eliminating the contribution of maternal IL-6 significantly decreases the level

of SOCS3 expression in MIA placentas, while eliminating the contribution of fetal/placental IL-6 has no significant effect. These data indicate that MIA leads to increases in maternally-derived IL-6 and subsequent downstream action in the placenta.

To examine whether maternally-derived IL-6 may act directly on spongiotrophoblasts to activate the JAK/STAT3 pathway, we used laser capture microdissection of the spongiotrophoblast layer and qRT-PCR to test for expression of IL-6 receptor components. On its target cells, IL-6 binds to its membrane-bound receptor, IL-6Rα, which associates with the signaling subunit of the receptor, gp130, to initiate intracellular signal transduction. Spongiotrophoblast cells from placentas of both saline and poly(I:C)-injected mothers indeed express IL-6Rα as well as gp130 (Fig. 4D) and are therefore likely to be capable of responding to placental IL-6. This offers the potential for direct IL-6 signaling in spongiotrophoblast cells and subsequent activation of STAT3.

In addition to IL-6, other factors also mediate STAT3 and STAT1 activation in the placenta after MIA. Both pSTAT3 and pSTAT1 staining are localized to parietal trophoblast giant cells (pTGCs) and mononuclear sinusoidal trophoblast giant cells (sTGCs) that reside in the junctional zone and labyrinth (Fig. S3-B,C). This staining is present with the same localization and abundance in placentas from WT and IL-6 KO mice injected with poly(I:C), suggesting that MIA-induced pro-inflammatory factors other than IL-6 mediate this effect. Furthermore, the staining is not blocked by co-injection of anti-IL-6 antibody and poly(I:C) in pregnant mothers (data not shown). Thus, MIA promotes widespread STAT3 and STAT1 activation in the fetal compartments of the placenta.

IL-6 specific signaling in response to MIA alters placental expression of factors that can influence fetal development.

The finding that IL-6 mediates STAT3 activation specifically in the spongiotrophoblasts inhabiting the fetal compartment of MIA placentas suggests that IL-6 may affect offspring development by altering expression of critical placental hormones and growth factors. In order to explore the downstream effects of MIA-induced STAT3 activation in the placenta, we conducted real time qRT-PCR for relevant placental factors using WT placentas from poly(I:C)or saline-injected mothers at 3 hours post-injection, a time at which pro-inflammatory cytokines are highly up-regulated. To assess the functional relevance of IL-6-mediated signaling in the placenta, we conducted similar real time qRT-PCR assays utilizing placentas from pregnant IL-6 KO mice injected with poly(I:C).

Maternal poly(I:C) injection leads to disruption of the GH-IGF axis in the placenta. Levels of GH protein are significantly reduced in MIA placentas (Fig. 5A-D), and there is also a corresponding decrease in IGFI expression, suggesting that reduced levels of GH may lead to decreased stimulation of IGFI production in the placenta (Forbes & Westwood, 2008). Furthermore, compared to saline-injected controls, placentas from poly(I:C)-injected mothers exhibit decreased IGFBP3 expression. IGFBP3 is a primary carrier binding protein responsible for increasing the half-life of IGFI, and its reduction is often associated with lower IGFI levels (Donahue & Beamer, 1993; De Benedetti, 2001; Liao et al., 2008). Reductions in both IGFI and IGFBP3 are absent in placentas from IL-6 -/mice injected with poly(I:C). In fact, placentas from MIA IL-6 KO mice exhibit increased expression of IGFI and IGFBP3, suggesting that basal

levels of IL-6 are also critical for regulation of IGFI and IGFBP3 expression. A similar effect is seen with levels of placental IGFI protein, where WT MIA placentas display a decreased concentration of IGFI compared to saline controls, while IL-6 KO MIA placentas display higher levels of IGFI. This provides further evidence that both basal IL-6 and MIA-induced IL-6 in the placenta play a role in suppressing IGFI and IGFBP3 production.

There are no differences between placentas from salineor poly(I:C)-injected mothers in the levels of GHRH, IGFII, IGF1R and IGFBP2, an inhibitory IGFI binding protein (Fig. 5A). Thus, MIA selectively reduces levels of placental GH and IGFI, two important hormones responsible for promoting embryonic development.

MIA also alters the expression of placental lactogen (PL) and pro-lactin-like proteins (PLPs) in the placenta. The PL and PLPs comprise families of prominent placenta-specific factors that regulate pregnancy, placental physiology and fetal development (Soares, 2004). Maternal poly(I:C) injection up-regulates placental PLP-K expression at 3 hours post-injection and this effect is abrogated in placentas from poly(I:C)-injected IL-6 -/mice (Fig. 6A, B). PLP-K is a placenta-specific protein that is widely expressed in the sTGCs of the labyrinth and in the spongiotrophoblast layer (Wiemers et al., 2003), an expression pattern that correlates precisely with IL-6-induced STAT3 activation in the placenta. Taken together, these data suggest that the activation of STAT3 driven by maternal IL-6 in response to MIA is responsible for the observed elevation in PLP-K expression. In addition, there is a trend towards increased expression of placental growth factor (PGF), a key a protein in angiogenesis and vasculogenesis in the placenta; placental lactogen 2 (PLII), a lactogen regulated by the inhibitory control of GH; as well

as PLP-F (Fig. 6A), a pro-lactin-like protein known to regulate hematopoiesis (Wiemers et al., 2003). No significant difference is seen in expression of PLI or PLP-E between saline and poly(I:C) placentas. Thus, MIA-induced IL-6 regulates expression of placenta-specific hormones that influence the maternal response to pregnancy and the regulation of fetal growth.

Discussion

The finding that IL-6 is critical for mediating the effects of MIA on the development of schizophreniaand autism-related endophenotypes in the mouse model offers the opportunity to trace the molecular and cellular pathways by which maternal infection increases risk for these neurodevelopmental disorders. We show that a single injection of rIL-6 into pregnant mice yields offspring with PPI, LI and open field deficits that are equal, if not more severe, than those seen in MIA offspring.

We find that maternally-derived IL-6 accounts for the increased pool of IL-6 in placentas from poly(I:C)-injected mothers. Potential sources of maternally-derived IL-6 in the placenta include circulating IL-6 in the maternal bloodstream and IL-6 that is secreted by maternal cells that reside in the placenta, such as decidual immune cells, stromal cells and endothelial cells. It is clear that maternal poly(I:C) injection increases the level of IL-6 and other pro-inflammatory cytokines in the maternal circulation (Gilmore et al., 2005; Meyer et al., 2008; Smith et al., 2007; Koga et al., 2009). However, our finding that IL-6 mRNA expression is dramatically increased in MIA placentas suggests that resident placental cells of maternal origin may also be a source of increased placental IL-6. Furthermore, we show that MIA increases the number of CD69-

expressing decidual macrophages, granulocytes and uNK cells in poly(I:C) placentas. CD69 is a marker of an early activation response, and its expression is associated with production of pro-inflammatory cytokines including IL-6 (Saito, 2000). Thus, following MIA, activated decidual leukocytes may contribute to the induction of IL-6 in the placenta (Lockwood et al., 2008). Some studies report that maternal stromal cells and endothelial cells in the placenta can also generate cytokines in response to other types of immune activation (Semer et al., 1991; Montes et al., 1995). Interestingly, in the CBA x DBA/2 model of miscarriage, early maternal poly(I:C) injection leads to increased expression of CD69, IFNγ and TNFα by uNK cells (Zhang et al., 2007). Altogether, these findings demonstrate that the MIA response is relayed to the maternal cells in the placenta, leading to increased levels of maternally-derived IL-6 directly at the materno-fetal interface.

Interestingly, compared to saline-injected controls, IL-6 protein is increased in amniotic fluid (AF) from poly(I:C)-injected mothers (Mandel et al., 2010). While the importance of the fetal placental component was stressed as the source of AF IL-6, it appears that much of the AF IL-6 is dependent on maternal IL-6 production. Eliminating the contribution of maternal IL-6 in poly(I:C)-injected mice decreases the concentration of AF IL-6 by over 14-fold compared to that observed in WT poly(I:C)-injected mice, and brings the final AF IL-6 concentration to a level lower than that observed in saline controls (Mandel et al., 2010). This indicates that maternal IL-6 production is critical for MIA-induced increases in AF IL-6. It will be important to compare AF from IL-6 +/offspring of IL-6 -/mothers with AF from IL-6 +/offspring of IL-6 +/mothers as a control.

We further demonstrate that maternally-derived IL-6 is responsible for activation of the JAK/STAT3 pathway specifically in the spongiotrophoblast layer, a fetal compartment of the placenta. It is likely that this STAT3 activation occurs by a direct effect of maternally-derived IL-6 on spongiotrophoblast cells, since we find that they express IL-6Rα and gp130. Moreover, paracrine signaling is a well-established mechanism of materno-fetal communication between distinct placental layers (Petraglia et al., 1996; Bischof et al., 2000; Lacey et al., 2002; Hess et al., 2006). The spatially-localized activation of STAT3 at the junctional zone of the placenta suggests that MIA induces maternally-derived IL-6 in the decidual layer (Fig. 7), which then signals in a paracrine manner to fetal spongiotrophoblast cells. It is important to note that, while a direct effect of IL-6 on placental cells is likely, we have not excluded the possibility that IL-6 may act as an upstream, indirect mediator of MIA effects on the placenta, and that IL-6 may act by so-called trans-signaling, or binding soluble IL-6Rα before complexing with membrane-bound gp130. Nonetheless, the maternal IL-6-dependent activation of the STAT3 pathway in spongiotrophoblast cells demonstrates a direct transfer of the MIA response from maternal to fetal cells.

Spongiotrophoblast cells arise just prior to mid-gestation and are a prominent source of endocrine factors throughout the latter half of gestation (Soares, 2004). We find an IL-6-dependent elevation in placental PLP-K expression after MIA, along with mild increases in several other pro-lactogen and pro-lactin-like-protein family members. As a recently discovered PLP, PLP-K is characterized as a placenta-specific protein produced primarily by spongiotrophoblast cells and also by sTGCs (Wiemers et al., 2003). The correlation of maternal-IL-6-dependent STAT3 activation in the

spongiotrophoblast with maternal-IL-6-dependent up-regulation of PLP-K suggests that modulation of PLP-K expression lies downstream of STAT3 activity. While its function is unclear, PLP-K possesses structural similarity to proliferin (PLF), a protein that binds mannose-6/IGFII complexes to regulate vasculogenesis and trophoblast proliferation. Thus, we demonstrate that MIA-induced maternal IL-6 alters expression of PLP-K and potentially other genes that encode endocrine or paracrine modulators of maternal and fetal cells.

Furthermore, we find an IL-6-dependent dysregulation of the GH-IGF axis in MIA placentas, characterized by decreased levels of GH and IGFI mRNA, with corresponding decreases in placental IGFI and IGFBP3 protein. The actions of GH are achieved through the stimulation of IGFI production in target tissues. In addition, GH regulates the activity of IGFI by altering the production of either facilitatory or inhibitory binding proteins, including the IGFI stabilizing protein, IGFBP3. This suggests that the decreased GH levels seen in MIA placentas leads to the observed downstream suppression of IGFBP3 and IGFI production. It is believed that IGFs in the maternal circulation do not enter the placenta, and therefore IGFs in the placenta are derived from the placental compartment itself (Kanai-Azuma et al., 1993).

We demonstrate that the changes in IGFI and IGFBP3 expression are mediated by IL-6. However, it is unclear whether decreases in placental GH and subsequent effects on IGF production are downstream of IL-6-specific STAT3 activation. IL-6 does modulate IGFI and IGFBPs in several tissues, including placenta and cord blood (De Benedetti et al., 2001; Street et al., 2009). Pro-inflammatory cytokines, including IL-6, decrease circulating and tissue concentrations of GH and IGFI (Lang et al., 2005). We observe that

IL-6-mediated STAT3 activation is associated with the expected IL-6-mediated increase in SOCS3 expression, along with other acute phase genes. Factors like SOCS play an important role in the down-regulation of GH and GH signaling (Herrington et al., 2000; Lang et al., 2005). Importantly, it is reported that IL-6 inhibits hepatic GH signaling through up-regulation of SOCS3 (Denson et al., 2003). As such, it is possible that, in MIA placentas, maternal IL-6-induced STAT3 activation and downstream sequelae lead to suppression of placental GH levels, disruption of IGFI production and further consequences on maternal physiology, placental function and fetal development.

Altered placental physiology and release of deleterious mediators to the fetus are important risk factors for the pathogenesis of neurodevelopmental disorders. Placental IGFI in particular regulates trophoblast function (Forbes & Westwood, 2008), nutrient partitioning and placental efficiency (Fowden et al., 2009). Moreover, altered IGFI levels are associated with intrauterine growth restriction (IUGR) and abnormal development (Laviola et al., 2008; Crossey et al., 2002). Animal models of IUGR and intrauterine infection, where the immune insult is confined to the uteroplacental compartment, highlight the key role of placental inflammation in perinatal brain damage, involving altered cortical astrocyte development (Bassan et al., 2010), white-matter damage (reviewed by Dammann & Leviton, 1997), microglial activation, cell death (Hutton et al., 2008) and reduced effectiveness of the fetal blood-brain-barrier (Yan et al., 2004). In addition, adult pathophysiology is subject to feto-placental “programming”, wherein molecular changes that occur prenatally reflect permanent changes that persist throughout postnatal life (Zhang et al., 2005; Seckl & Holmes, 2007; Merlot et al., 2008; Bilbo & Schwarz, 2009; Barker et al., 2010a,b; Frost & Moore, 2010). Interestingly, placental

responses to maternal insults can potentiate sexually dimorphic effects on fetal development (Clifton, 2005; Mueller & Bale, 2008).

Obstetric complications are linked to schizophrenia risk (reviewed by Preti et al., 2000) and to the treatment responses of schizophrenic individuals (Alivir et al., 1999). Notably, a greater occurrence of placental trophoblast inclusions was observed in placental tissue from children who develop autism spectrum disorder (ASD) compared to non-ASD controls (Anderson et al., 2007). Chorioamnionitis and other obstetric complications are significantly associated with socialization and communication deficitis in autistic infants (Limperopoulos, 2008). The characterization of placental pathophysiology and obstetric outcome in ASD and schizophrenic individuals will be useful for the identification of molecular mechanisms that underlie these disorders and for potential biomarkers for early risk diagnosis.

In addition to the observed effects of IL-6 on placental physiology and its downstream effects on fetal brain development and postnatal growth, direct effects of IL-6 on the fetal brain are also likely. Maternal IL-6 can potentially cross the placenta and enter the fetus after MIA (Dahlgren et al., 2006). Furthermore, IL-6 mRNA and protein are elevated and STAT3 is phosphorylated in the fetal brain itself following MIA (Gilmore et al., 2005; Meyer et al., 2008; Hsiao & Patterson, 2009), raising the obvious possibility that IL-6 acts directly on the developing brain to influence astrogliosis, neurogenesis, microglial activation and/or synaptic pruning (Conroy et al., 2004; Gilmore et al., 2004; reviewed in Deverman & Patterson, 2010). However, recall that the identification of IL-6 as a critical mediator of MIA is based on maternal co-injection of poly(I:C) and anti-IL-6 blocking antibody, in addition to experiments inducing MIA in

IL-6 KO animals. As such, in considering which pool(s) of IL-6 (e.g. maternal, placental, fetal brain, fetal periphery) is the “critical mediator”, it will be important to understand the potential interaction between maternal IL-6 and fetal brain IL-6 expression. While we believe that the endocrine changes triggered by maternal-IL-6 signaling in the placenta reported here are important for fetal growth, it will be crucial to assess the potential impact of these placental changes on offspring behavior and neuropathology. We are currently exploring the effects of MIA in targeted IL-6Rα KOs in order to tie tissueand cell-specific IL-6 activity to the manifestation of schizophreniaand autism-related endophenotypes.

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Methods

Generation of animals.

Female C57BL/6J mice (Charles River; Wilmington, MA) were obtained from the Caltech breeding facility and housed under standard laboratory conditions. Mice were mated overnight and the presence of a vaginal plug on the following morning was noted as day E0.5.

IL-6 KO mice.

IL-6 KO mice, strain B6.129S2-IL6tm1Kopf/J, were obtained from Jackson Laboratory (Bar Harbor, ME). IL-6 -/females were mated with IL-6 +/males, and IL-6 +/females were mated with IL-6 -/males. For assays involving placental samples, a small amount of tissue from the corresponding fetus was used for genotyping.

MIA.

Pregnant C57BL/6J or IL-6 KO mice were injected on E12.5 with saline, poly(I:C), or recombinant IL-6 (rIL-6). For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO) was freshly dissolved in saline and administered i.p. at 20 mg/kg based on the weight of the poly(I:C) itself, not including the total weight of the potassium salts. Control mice were injected with saline alone at 5 ml per gram body weight. For rIL-6 injections, 5 mg carrier-free, recombinant mouse IL-6 (eBioscience; San Diego, CA) was freshly dissolved in 150 ml saline and injected i.p.

Behavioral testing.

After injection on E12.5, each pregnant mouse was single-housed. At 6 weeks of age, offspring were behaviorally tested for PPI; at 8 weeks, for LI, and at 10 weeks, for open field exploration, according to methods described by Smith et al., 2007.

Measurement of placental IL-6, growth hormone and IGFI.

After injection on E12.5, wild type (WT) and IL-6 KO mice were sacrificed at 3, 6 or 24 hours by an overdose of sodium pentobarbital (Nembutal). Placentas were dissected from the uterine horn and washed in PBS prior to snap-freezing in liquid nitrogen and storage at -80°C. To generate cell lysates, each placenta was placed in 1 ml of cell lysis buffer (50 mM Tris-HCl (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 1% BSA, and 1 EDTA-free protease inhibitor cocktail tablet/10 ml buffer) (Roche Applied Sciences; Indianapolis, IN). Each tissue was homogenized on ice using a syringe fitted with an 18G needle and then sonicated for 5 seconds at 10 mV. The homogenates were centrifuged at 4°C at 13,000 rpm for 20 minutes, and the supernatants aliquotted and frozen at -80°C until assayed. ELISAs for IL-6 (eBioscience), GH (Millipore; Billerica, MA), and IGFI (R&D Systems; Minneapolis, MN) were performed according to the manufacturers' instructions and analyzed against cell lysis buffer negative controls. Total protein was measured by BCA assay (Thermo Scientific; Rockford, IL) according to the manufacturer's instructions.

Measurement of placental gene expression by real-time PCR.

Placentas were quickly dissected from the uterine horn and washed in PBS prior to preservation in 1 ml TRIzol solution (Invitrogen; Carlsbad, CA). Tissues were passed

through an 18G needle and sonicated for two rounds of 3 seconds at 10 mV separated by incubation on ice. Homogenates were processed by chloroform extraction and washing with 70% ethanol according to standard procedures. RNA was further purified by applying the cleared lysate to an RNeasy mini column (Qiagen; Valencia, CA), and an on-column DNA digestion was performed according to the manufacturer's protocols. Samples were assayed using the 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and confirmed to contain high integrity RNA (RIN > 8). 5 mg RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Biorad; Hercules, CA). Resultant cDNA was purified using a PCR purification kit (Qiagen) and eluted in 50 ml PCR-grade water (Roche). Gene expression was measured using SYBR Green master mix with Rox passive reference dye (Roche) on the ABI 7300 Real Time PCR system. Target gene expression was normalized against beta-actin transcript, and data are expressed as ratios of gene expression of poly(I:C) to saline samples. The primers used were adapted from the Primerbank database (Spandidos et al., 2010).

Immunohistochemistry.

Placentas were dissected and washed in PBS prior to fixation for 1.5 hours in 4% paraformaldehyde at 4°C and cryopreservation in 30% sucrose overnight at 4°C. After equilibration for 1 hr in Tissue-Tek OCT (Sakura Finetek; Torrance, CA), each placenta was hemi-sected laterally prior to freezing. Placentas were cut in 12 mm sagittal sections, with 8 medial sections spanning approximately 576 mm of tissue (every sixth section) taken from each placenta for each histological stain. Sections were stained for phospho-STAT3 and phospho-STAT1 (Cell Signaling; Danvers, MA) according to standard

procedures. Briefly, antigen retrieval was conducted for 30 minutes in a 95°C water bath in 10 mM sodium citrate pH 6.0, for pSTAT3, and 1 mM EDTA pH 9.0, for pSTAT1. Slides were equilibrated to room temperature, washed and incubated for 10 minutes in 3% H_2O_2 in methanol. After washing, tissues were processed using Vectastain Elite ABC peroxide kits (Vector Labs; Burlingame, CA) according to the manufacturer's instructions, with overnight incubation of primary antibody at 4°C. Staining was developed using ImmPACT DAB substrate (Vector Labs) and mounted in Vectamount aqueous mounting medium (Vector Labs).

Flow cytometry of placental leukocytes.

The decidua was dissected carefully from the labyrinth layer and washed in PBS on ice. 7-8 tissues from each litter were pooled in RPMI 1640 medium with 10% FBS and 1% penicillin/streptomycin and subjected to mechanical disruption by passage through a syringe fitted with a 16G needle followed by a syringe with an 18G needle. Tissue suspensions were then enzymatically disrupted with 0.1% collagenase for 30 minutes at 37°C, followed by 0.25% trypsin for 10 minutes at 37°C. Cell suspensions were washed with 3 volumes of RPMI and passed through 22G and 25G needles and subjected to 2 rounds of RBC lysis and filtration through 40 mm cell strainers. Cell counts were performed on a hemocytometer, and 1 million cells were aliquotted for each subsequent reaction. Single cell suspensions were treated with anti-mouse CD16/CD32 Fc block (eBioscience) for 10 minutes on ice prior to staining in CBSS with the following antibody conjugates: DBA-lectin-FITC (Sigma), CD69-PE (eBioscience), Ter119-PerCP-Cy5.5 (BioLegend; San Diego, CA), CD4-FITC (BioLegend), CD8-FITC (Biolegend), Gr-1-

APC (BioLegend), B220-FITC (BioLegend) and CD11b-APC (BioLegend). Flow cytometry was performed using the FACSCalibur cytometer (BD Biosciences; San Jose, CA), and data were analyzed using FlowJo software (TreeStar Inc.; Ashland, OR) and presented as percent frequency of the parent population (non-erythroid (Ter119-) cells).

Laser capture microdissection (LMD).

Placentas were dissected, washed in PBS and immediately snap-frozen in liquid nitrogen. Tissues were then embedded in Tissue-Tek OCT and cut into 12 mm sections on PALM Membrane Slides (PALM Microlaser Technologies; Germany). Cryostat blades and workstations were cleaned with RNaseZap (Applied Biosystems; Austin, TX) to prevent RNA degradation. 6 medial sagittal sections were stained with hematoxylin QS (Vector Labs) and used for laser microdissection. Duration was limited to < 30 minutes per slide to prevent RNA degradation. For each placental section, the spongiotrophoblast layer was localized by morphology under the Axio Observer.Z1 confocal microscope (Zeiss; Thornwood, NY). Conservative regions of interest encompassing approximately 100 cell nuclei were microdissected (energy: 40-48; focus: 71) using the PALM Microbeam system and PALMRobo software 4.3 (Zeiss) and immediately expelled into an AdhesiveCap 500 microfuge tube (Zeiss). RNA isolation was performed immediately as described above, with the RNeasy Micro Plus kit (Qiagen). Genomic DNA was removed using gDNA eliminator columns (Qiagen). Total RNA was amplified and reverse transcribed using the Quantitect Whole Transcriptome Amplification kit (Qiagen). 100 ng cDNA was used for qPCR, according to the methods described above. No differences in relative ß-actin, IL-6Rα and gp130 expression were observed between saline and

poly(I:C) samples, and treatment groups were therefore merged for greater statistical power.

Statistical analysis.

Statistical analysis was performed with Prism software (Graphpad Software; La Jolla, CA). The statistical significance of differences between two treatment groups was assessed using the Student's t-test, and differences among multiple groups was assessed using one-way ANOVA followed by Bonferroni post-hoc tests.

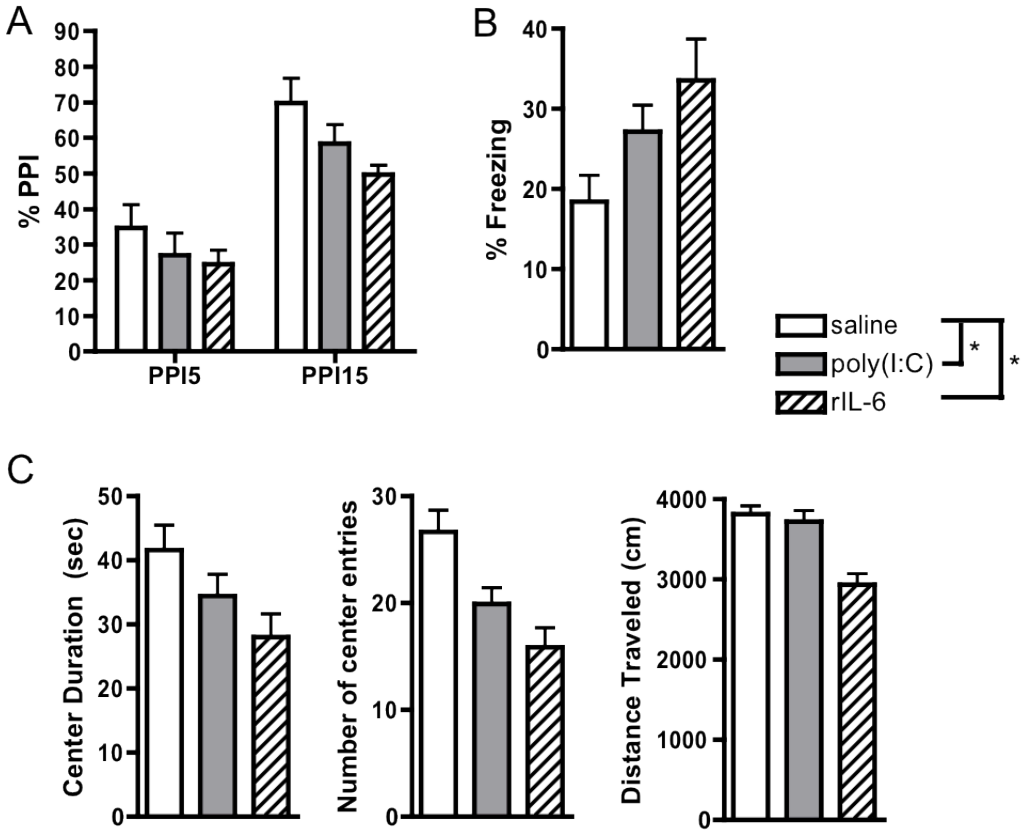

Fig. 1. Maternal IL-6 exposure yields offspring with behavioral abnormalities analogous to those seen in MIA offspring. A. Compared to controls, offspring of mice treated with rIL-6 or poly(I:C) display a PPI deficit [$F(2, 74)=4.40$, $^*p < 0.05$]. B. rIL-6 and poly(I:C) offspring display increased freezing in response to the conditioned acoustic cue during LI testing, as well as decreased LI compared to saline controls when measured against the NPE group [% freezing = 64.49 ± 6.05 (mean ± SEM)] [$F(2, 41)=3.261$, $^*p < 0.05$]. NPE poly(I:C) and saline offspring display no significant difference in freezing response. C. Compared to controls, offspring of rIL-6 or poly(I:C)-injected mothers exhibit fewer entries into the center of the open field [$F(2, 92)=8.596$; $p < 0.0005$] and shorter duration spent in the center field [$F(2, 92)=3.140$; $^*p < 0.05$]. rIL-6 offspring also present shorter total distance traveled in the open field [$F(2, 92)=12.81$; $p < 0.0001$].

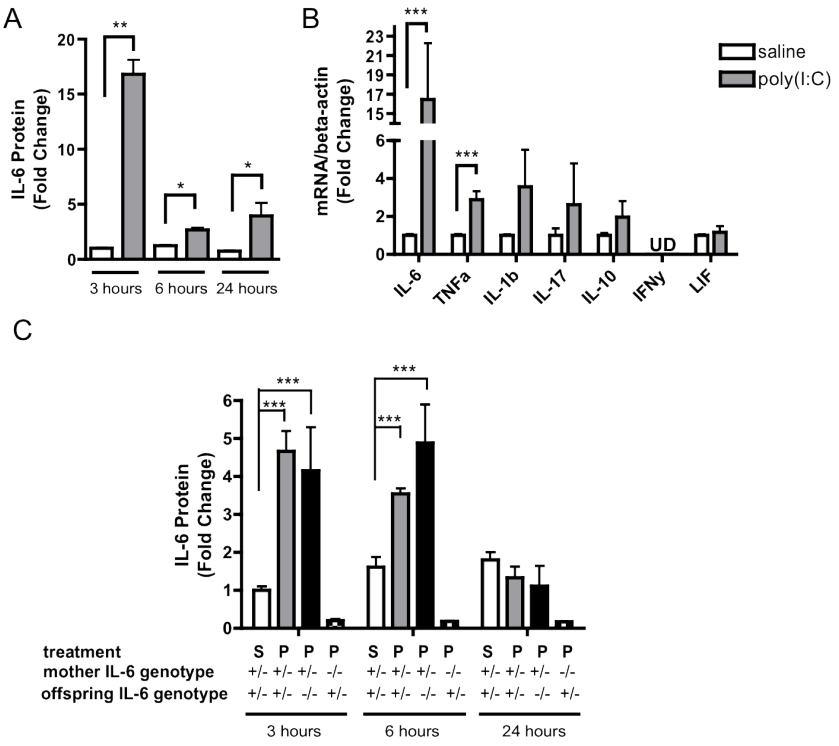

Fig. 2. MIA upregulates maternally-derived IL-6 in the placenta. A. Compared to controls, placentas from poly(I:C)-injected mothers exhibit increased IL-6 protein at 3, 6 and 24 hours post-injection [n= 9 per treatment per time point (from 3 independent litters); * $p < 0.05$, ** $p < 0.001$]. B. At 3 hours post-injection, compared to saline controls, placentas from poly(I:C)-injected mothers also display increased mRNA expression of pro-inflammatory cytokines, including a markedly elevated level of IL-6 [n=9 per treatment group (from 3 independent litters); ***$p < 0.0001$]. C. Eliminating the contribution of fetally-derived IL-6 has no effect on total placental IL-6 levels, whereas eliminating the contribution of maternally-derived IL-6 completely diminishes total placental IL-6 to levels below those seen in saline controls at both 3 hours and 6 hours post-injection. This indicates that basal and MIA-induced placental IL-6 is maternally-derived. [$F(3,80)=24.89$; ***$p < 0.0001$].

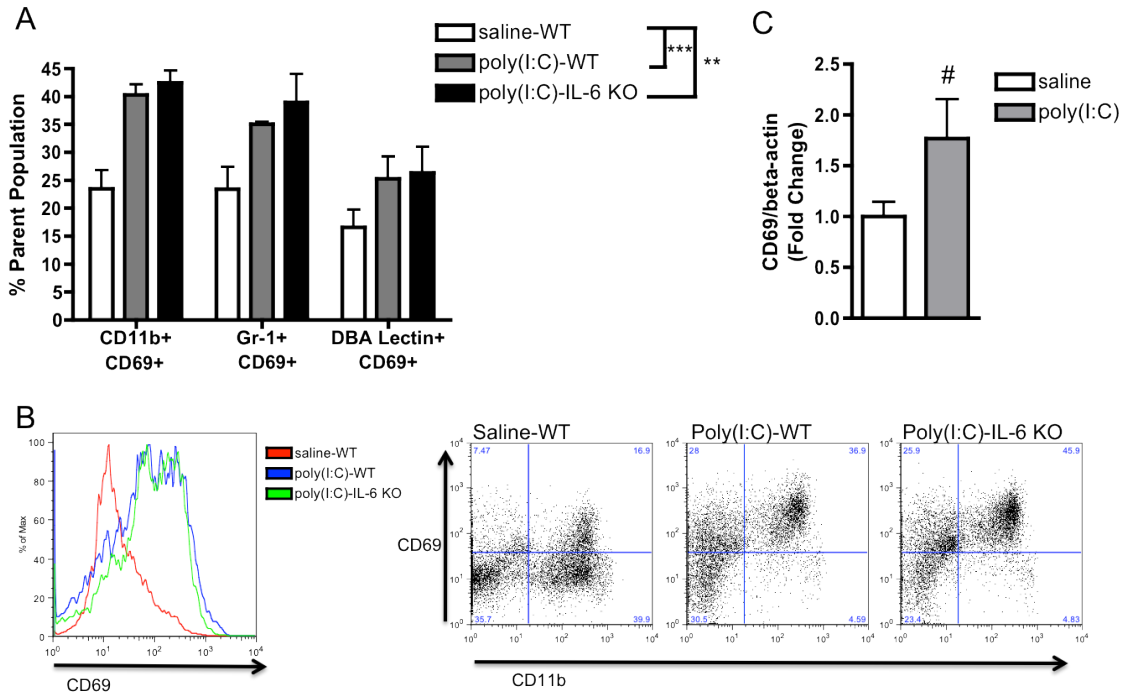

Fig. 3. MIA activates decidual leukocytes to express CD69. A. Compared to controls, placentas from poly(I:C)-injected mothers harbor increased numbers of CD69-expressing CD11b+, Gr-1+ and DBA lectin+ cells [$F(1,12)=24.76$, ***$p=0.0003$]. These are similarly increased in poly(I:C)-injected IL-6-/mothers, suggesting that this effect occurs independently of IL-6 action [$F(1,12)=21.87$, **$p=0.0005$]. B. Representative flow cytometry spectra show increased total CD69+ cells in poly(I:C) decidual suspensions (left) and increased CD69+ CD11b+ cells from a non-erythroid parent population (right) C. Increased activation of decidual leukocytes corresponds with a trending increase in total CD69+ gene expression (# $p = 0.08$)

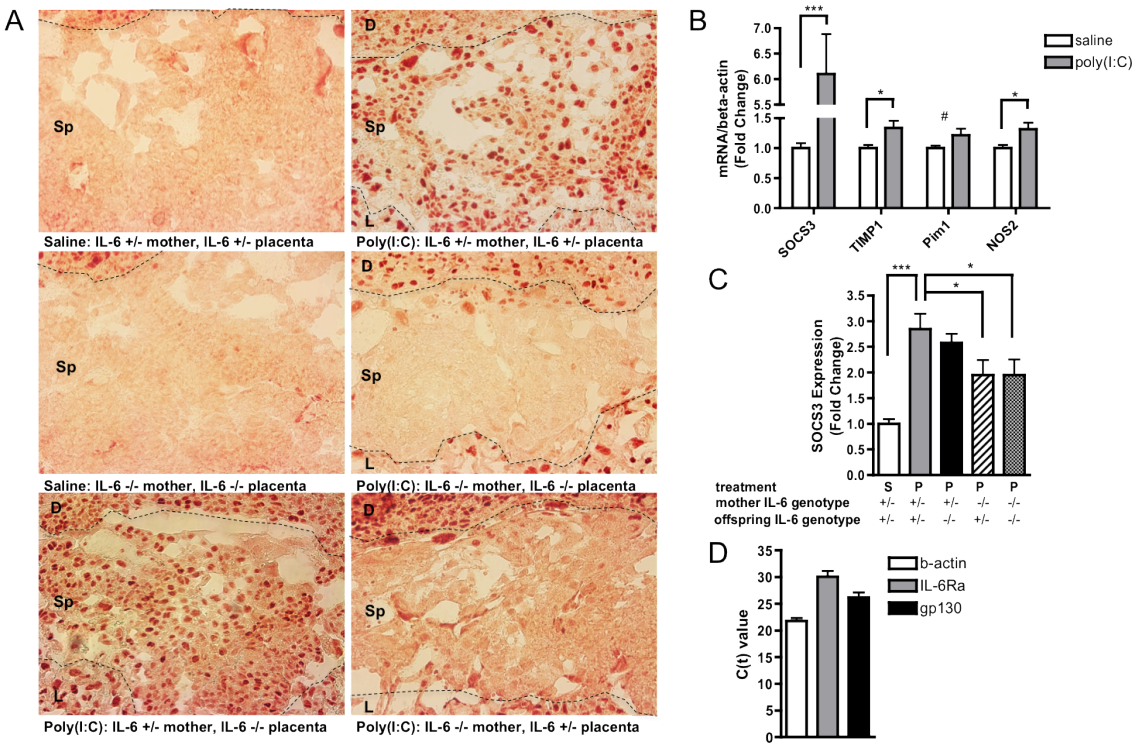

Fig. 4. **Maternally-derived IL-6 activates the JAK/STAT3 pathway in the fetal placental compartment in response to MIA.** A. Representative sections of the spongiotrophoblast layer of the placenta (with decidua along the top edge and labyrinth along the bottom edge of each image) show positive pSTAT3 staining when placentas are isolated from poly(I:C)-injected mothers. Eliminating the contribution of fetally-derived IL-6 has no effect on pSTAT3 staining, while eliminating the contribution of maternally-derived IL-6 completely abrogates pSTAT3 staining in the spongiotrophoblast. [n=5-7 placentas per treatment group per genotype pair (from 3-5 independent litters)]. *Dotted lines = boundary between decidua and spongiotrophoblast layers (upper) and boundary between spongiotrophoblast and labyrinth (lower); D=decidua, Sp=spongiotrophoblast, L=labyrinth*; B. MIA-induced JAK/STAT3 activation is accompanied by increased expression of the downstream acute phase genes, SOCS3, TIMP1, Pim1 and NOS2, in

poly(I:C) placentas [n=9 per treatment group (from 3 independent litters); ***$p < 0.0001$, *$p < 0.05$, # $p = 0.07$]. C. Eliminating the contribution of maternally-derived IL-6 reduces placental SOCS3 induction [n=9 per treatment group per genotype pair (from 3 independent litters); ***$p < 0.0001$, *$p < 0.05$]. D. Murine spongiotrophoblast cells express IL-6Rα and gp130 mRNA, indicating that they may respond directly to placental IL-6 activation. [n=3 saline, 3 poly(I:C}; merged].

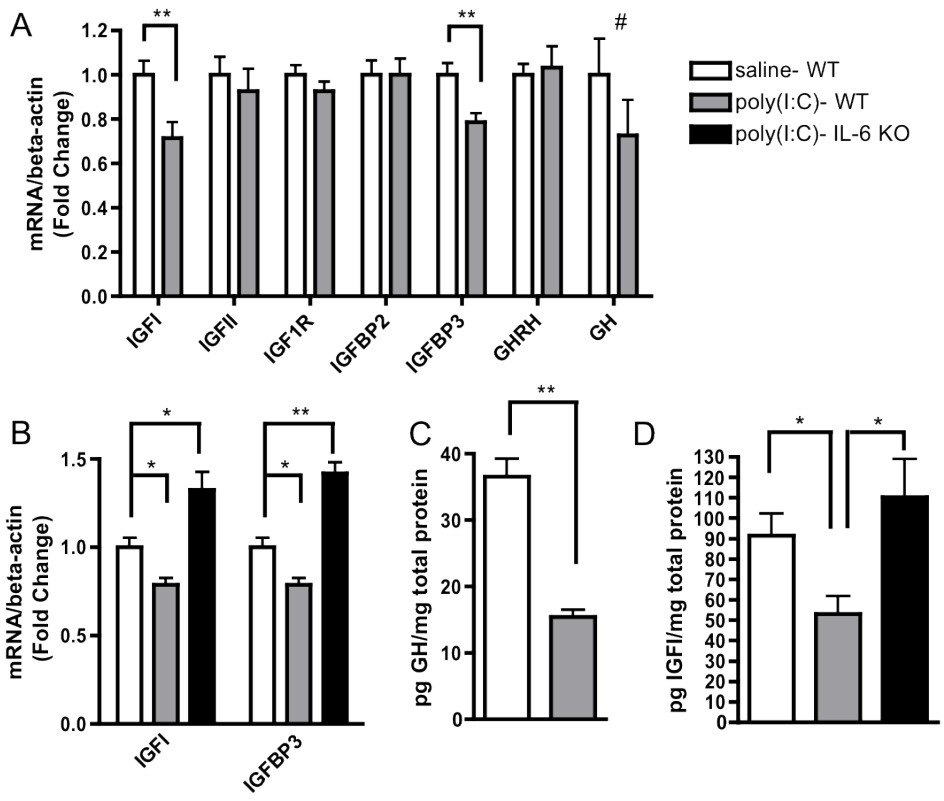

Fig. 5. **MIA-induced IL-6 action reduces placental IGFI and IGFBP3.** A. Compared to saline controls, placentas from poly(I:C)-injected mothers have significantly reduced expression of IGFI and IGFBP3 mRNA and a trending decrease in GH expression [n=9 per treatment group (from 3 independent litters); $^{**}p < 0.001$, $\# p = 0.24$]. B. The deficits in IGFI and IGFBP3 expression observed in MIA placentas require IL-6 production [IGFI: $F(2,23)=15.95$; $^{*}p < 0.05$; IGFBP3: $F(2,23)=37.77$; $^{**}p < 0.001$]. C. MIA placentas exhibit significantly decreased GH protein [n=7 placentas per treatment group (from 3 independent litters); $^{**}p < 0.001$]. D. MIA placentas exhibit significantly decreased IGFI protein, and this deficiency is dependent on IL-6 production [$F(2, 17)=5.908$; $^{*}p < 0.05$].

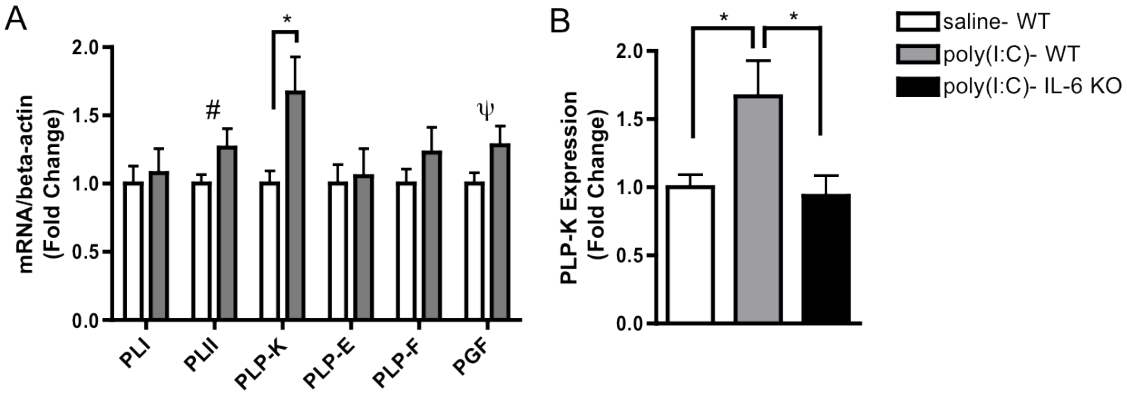

Fig. 6. **MIA-induced IL-6 action increases PLP-K and related growth factor gene expression.** A. Placentas from poly(I:C)-injected mothers have significantly elevated expression of PLP-K and trending increases in PGF and PLII [n=9 per treatment group (from 3 independent litters); *$p < 0.05$, # $p = 0.10$, ψ $p = 0.11$]. B. The upregulation of PLP-K expression observed in MIA placentas require IL-6 action [$F(2,23)=5.033$; *$p < 0.05$].

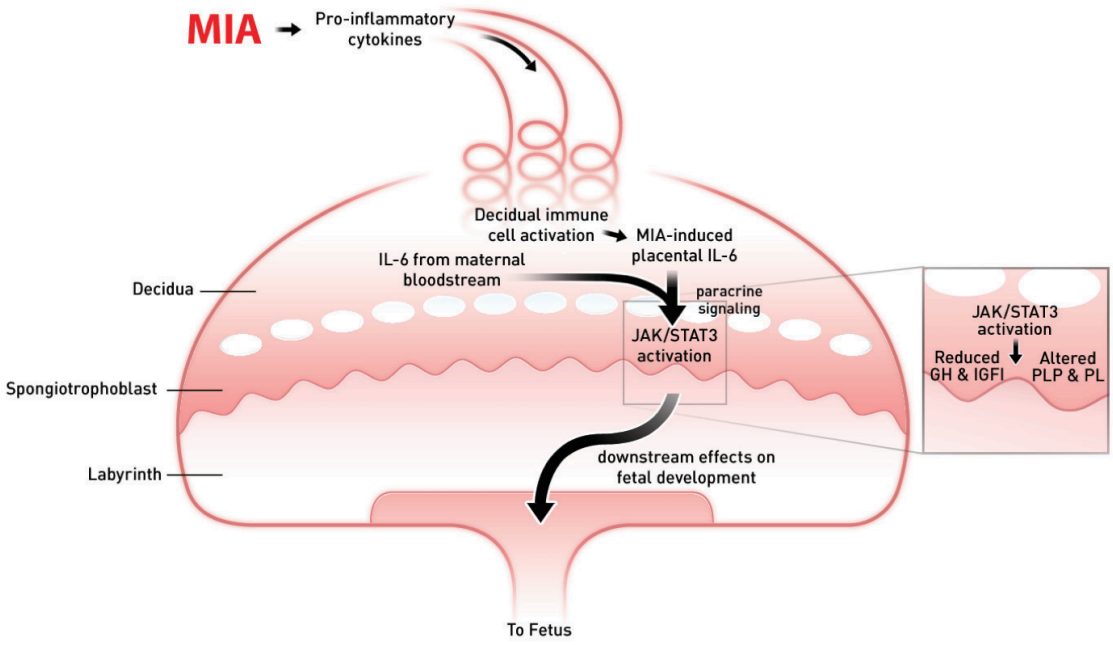

Fig. 7. Model of MIA-induced IL-6 effects on the placenta. Maternal injection of poly(I:C) leads to the activation of the maternal immune system via a TLR3-mediated anti-viral response. Pro-inflammatory factors, including IL-6, are secreted by activated TLR3+ cells into the maternal bloodstream. As maternal blood circulates continuously through the placenta, IL-6 and soluble pro-inflammatory factors increase in the spiral arteries that descend through the decidua and spongiotrophoblast, as well as in the maternal blood spaces of the labyrinth, and circulate back up into the maternal compartment. Resident immune cells in the decidua are activated by maternal cytokines and other signaling factors to express CD69, and they propagate the inflammatory response by further cytokine release. IL-6 derived from decidual cells acts in a paracrine manner on target cells in the spongiotrophoblast layer. Ligation with the cognate receptor IL-6Ra and gp130 leads to signal transduction resulting in JAK/STAT3 activation and downstream changes in gene expression. Increases in acute phase proteins, such as SOCS3, down-regulate placental GH production and signaling. This leads to reduced IGFBP3 and IGFI. Global changes in STAT3 activation in the spongiotrophoblast layer

alter the production of placenta-specific PLP and pro-lactin proteins. These changes in endocrine factors lead to acute placental pathophysiology and subsequent effects on fetal development.

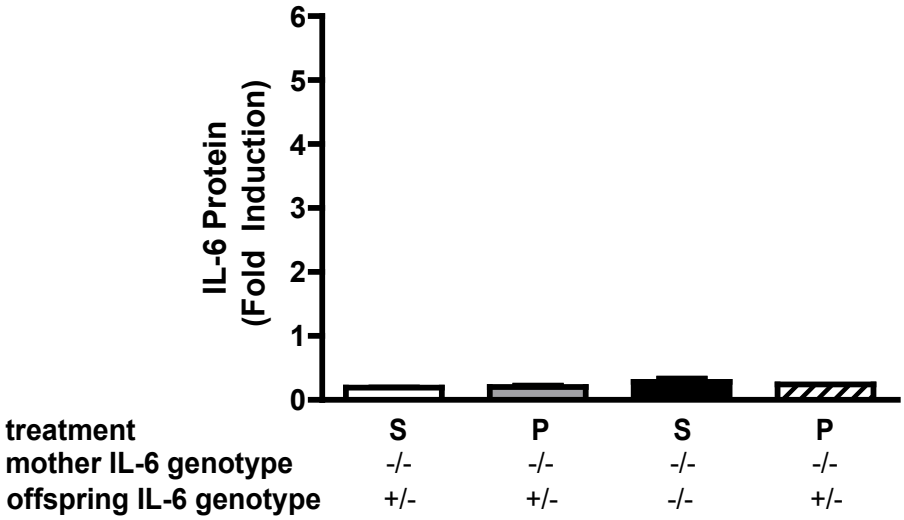

Fig. S1. **Fetally-derived IL-6 contributes negligible amounts of IL-6 protein to the placenta after MIA.** Placentas were collected to assess levels of placental IL-6 by ELISA. A low-level absorbance signal is observed in IL-6 -/placentas from IL-6 -/mothers, indicating a degree of non-specific protein binding during the experimental procedure. All placentas from salineor poly(I:C)-injected IL-6 -/mothers exhibit similarly negligible amounts of absorbance signal.

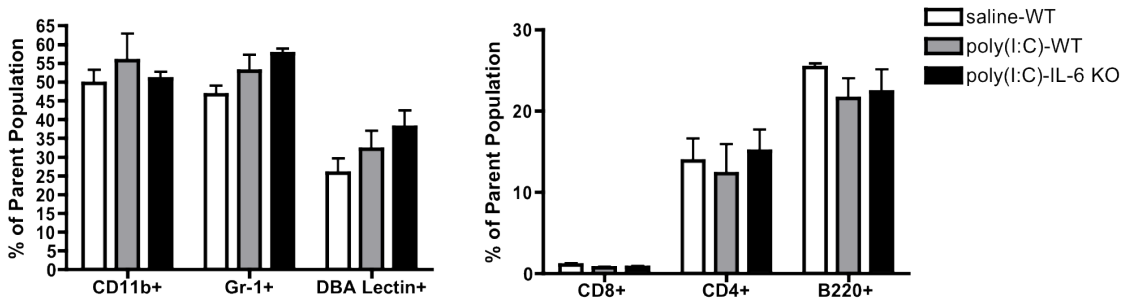

Fig. S2. **MIA has no affect on percentages of decidual immune cell subtypes.** Compared to controls, placentas from poly(I:C)-injected mothers have no significant difference in the percentages of macrophages, granulocytes, uNK cells, CD4+ T cells, CD8+ T cells or B cells.

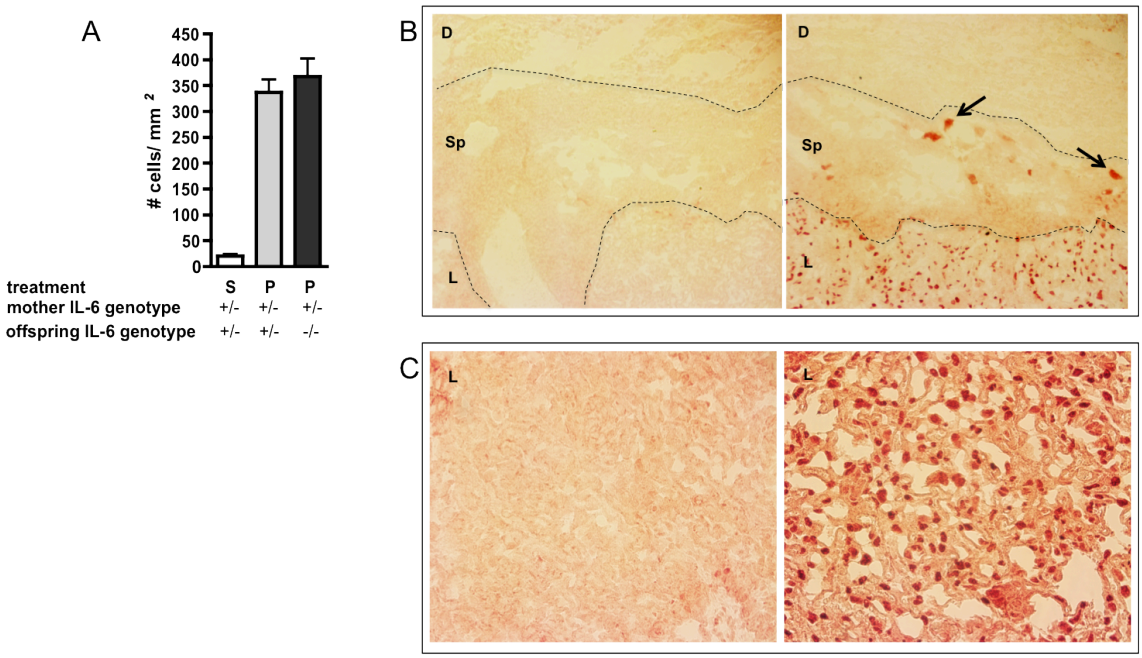

Fig. S3. **MIA induces phosphorylation of STAT1 and STAT3 in sTGCs and pTGCs in an IL-6-independent manner.** A. IL-6 +/and IL-6 -/placentas from poly(I:C)-injected IL-6 +/mothers display no significant difference in the number of pSTAT3-positive spongiotrophoblast cells. Thus, fetally-derived IL-6 contributes little to MIA-induced STAT3 activation. B. Representative images taken at 20X magnification depict saline placenta (left) and MIA placenta (right). Compared to saline controls, MIA placentas (right) stain positively for pSTAT1 in sTGCs and pTGCs (arrows). *Dotted lines = boundary between decidua and spongiotrophoblast layers (upper) and boundary between spongiotrophoblast and labyrinth (lower); D=decidua, Sp=spongiotrophoblast, L=labyrinth*; C. Representative images taken at 40X magnification depict pSTAT3 staining in saline labyrinth (left) and MIA labyrinth (right). Compared to saline controls, MIA placentas stain positively for pSTAT3 in sTGCs.

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Chapter 7

Effects of maternal immune activation on gene expression patterns in the fetal brain

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Abstract

We are exploring the mechanisms underlying how maternal infection increases the risk for schizophrenia and autism in the offspring. Several mouse models of maternal immune activation (MIA) were used to examine the immediate effects of MIA induced by influenza virus, poly(I:C) and IL-6 on the fetal brain transcriptome. Our results indicate that all three MIA treatments lead to strong and common gene expression changes in the embryonic brain. Most notably, there is an acute and transient up-regulation of the alpha, beta and gamma crystallin gene family. Furthermore, levels of crystallin gene expression are correlated with the severity of MIA as assessed by placental weight. The overall gene expression changes suggest that the response to MIA is a neuroprotective attempt by the developing brain to counteract environmental stress, but at a cost of disrupting typical neuronal differentiation and axonal growth. We propose that this cascade of events might parallel the mechanisms by which environmental insults contribute to the risk of neurodevelopmental disorders such as schizophrenia and autism.

Introduction

Maternal infection is a risk factor for schizophrenia and autism. In the case of schizophrenia, a wide variety of infections during pregnancy (viral, bacterial, parasitic) are associated with increased risk for this disorder in the offspring. Summing these risks, Brown and Derkits (2010) estimate that >30% of schizophrenia cases would be prevented if infection could be averted in pregnant women. The fact that such a diverse set of pathogens is associated with risk suggests that it is the mother's response to the infection that is critical for altering fetal brain development. In fact, the maternal response during gestation (elevated cytokines and anti-pathogen antibodies) is associated with the increase in schizophrenic outcome in the offspring(A. S. Brown & Derkits, 2010). Similarly for autism, a study of >10,000 cases in the Danish Medical Registry revealed an association between viral or bacterial infection in the mother and increased risk for the offspring(Atladottir et al., 2010). Also similar to schizophrenia are findings of an increased risk for autism in the offspring if particular cytokines or a chemokine are elevated in maternal serum or amniotic fluid (Abdallah et al., 2011; Goines et al., 2011). The observation of a significantly higher concordance in dizygotic twins than in non-twin siblings also suggests the importance of the maternal-fetal environment in autism (Hallmayer et al., 2011; Rosenberg et al., 2009).

To mimic the environmental risk for development of schizophrenia and autism, several animal models for maternal immune activation (MIA) have been used successfully (Patterson, 2009). First, intranasal instillation of human influenza virus in pregnant mice or non-human primates induces a moderate but sublethal infection, and the offspring display a series of histological and molecular abnormalities in the hippocampus

and cortex (Fatemi, Cuadra, El-Fakahany, Sidwell, & Thuras, 2000; Fatemi et al., 1999). Young and adult mouse MIA offspring (Shi, Fatemi, Sidwell, & Patterson, 2003) also exhibit a cerebellar neuropathology that is commonly found in autism (Amaral, Schumann, & Nordahl, 2008; Palmen, van Engeland, Hof, & Schmitz, 2004) Furthermore, adult mice born to infected mothers display behavioral abnormalities that are relevant to both schizophrenia and autism, including deficits in social interaction, prepulse inhibition (PPI), and open field and novel object exploration (Shi, et al., 2003), as well as heightened responsivity to a hallucinogen (Moreno et al., 2011). Second, MIA can also be induced in the absence of pathogens by injecting pregnant rats, mice or monkeys with the synthetic dsRNA, poly(I:C) to mimic a viral infection, or with lipopolysaccharide (LPS) to mimic a bacterial infection. Overall, the behavioral results from the maternal infection, poly(I:C) and LPS models is consistent, with many results being reproduced in both rats and mice (Meyer & Feldon, 2010; Patterson, 2009, 2011). Molecular and cellular studies of adult offspring of poly(I:C)-treated rodents reveal abnormalities that are clearly relevant to schizophrenia, such as increased levels of $GABA_A$ receptor α2 immunoreactivity, dopamine hyperfunction, delay in hippocampal myelination, reduced NMDA receptor expression in hippocampus, reduced numbers of reelin and parvalbumin-positive cells in prefrontal cortex, reduced dopamine D1 and D2 receptors in prefrontal cortex and enhanced tyrosine hydroxylase in striatal structures (Meyer et al., 2008). Third, MIA can be induced more directly by a single injection of the cytokine IL-6 in pregnant mice (Smith, Li, Garbett, Mirnics, & Patterson, 2007). This approach is based on findings that this injection yields offspring displaying many of the same behavioral abnormalities found in offspring of influenza-infected or poly(I:C)-

treated dams. Moreover, co-injection of an anti-IL-6 antibody blocks the effects of poly(I:C), yielding offspring with normal behavior. The critical importance of IL-6 in MIA is further shown by the finding that poly(I:C) injection in IL-6 knockout mice also yields offspring with normal behavior (Smith, et al., 2007). Flavonoids that block JAK/STAT signaling downstream of the IL-6 receptor also block the induction of abnormal behaviors by maternal IL-6 injection (Parker-Athill et al., 2009). Maternal IL-6 is also critical for the endocrine changes in the placenta induced by MIA (Hsiao & Patterson, 2011)

However, the immediate effects of MIA on the fetal brain are not well understood. It is critical to examine the very early effects because the rise in IL-6 caused by maternal IL-6 injection is transient, as is the effect of injecting the anti-IL-6 blocking antibody in the pregnant dam. These acute effects nonetheless lead to permanent changes in the behavior of the offspring. To gain a better understanding of the molecular events that take place in the MIA-exposed developing brain, we examined the transcriptome changes associated with three different MIA models, and identify the critical early mediators that may contribute to the emergence of behavioral symptoms in MIA offspring.

Results

Transcriptome changes in flu-, poly(I:C)-, and rIL-6-treated embryonic brains

Five pregnant dams were treated with a single, nasal installation of flu virus at E9.5. The embryos were dissected on E12.5, the time of peak maternal IL-6 expression,

and brains were collected. A single treatment of poly(I:C), rIL-6 or saline was administered to four pregnant dams per group at E12.5. The embryonic brains from these treatments were collected 3h after injection. RNA was isolated from the entire brain of individual embryos. We obtained 31 to 36 embryos per treatment, which totaled 138 embryonic brain samples. For microarray analysis, embryonic brains from each litter were pooled into a single sample. Using a 30% expression difference from saline-injected controls and a statistical significance of $p<0.05$, we identified 256 genes differentially expressed in the flu model, 294 in the poly(I:C) model, and 195 in the IL-6 model. Examining all of the differentially expressed genes in a two-way (samples and genes) unsupervised hierarchical clustering of the gene expression intensities, the four experimental groups separate into distinct clusters (**Fig. 1A**) that correspond to the different MIA treatments, with the main clusters separating the saline-treatment from all three MIA-treatments. Interestingly, the majority of the genes altered in the poly(I:C) and IL-6 treatments were up-regulated, while there were as many down-regulated genes in the flu treatment, which might reflect secondary expression changes that occurred during the 3 day period following the influenza virus exposure.

Next, we tested if the transcriptome changes showed a common expression pattern across the three different MIA treatments. We created three groups of the most differentiallyexpressed genes: 256 genes in the flu model, 294 in the poly(I:C) model and 195 in the IL-6 model. The mRNA signature of the 256 differentially expressed transcripts in the flu model was assessed in the poly(I:C) and rIL-6 transcriptomes. We found that, as a group, the flu-model transcripts are also significantly changed in the poly(I:C)-model and IL-6-model for MIA ($r=0.88$, $p=1.8E-84$ and $r=0.85$, $p=5.95E-72$

respectively) (Fig. 1B,C). Likewise, the 195 differentially expressed genes in the IL-6-MIA group show a significant expression difference in the Flu-model and poly(I:C)-model brains (r=0.85, p=1.42E-55 and r=0.85, p=1.06E-54 respectively) (Fig. 1D,E), and the 294 transcript changes in the IL-6 group are also significantly present in the fluand poly(I:C)-treatments (r=0.59, p=8.78E-29 and r=0.59, p=3.9E-29 respectively) (Fig. 1F,G). This is a strong indication that all three treatments affect similar molecular processes, although their effects might be somewhat different because of the timing difference in flu exposure and potential downstream signaling differences. A comprehensive list of the differentially expressed genes and their expression levels is provided in Supplemental Material 2.

Of the transcripts with "most changed" expression (>30% expression change and $p<0.05$ in all three treatments), 41 genes were altered in both IL-6 and flu treatment, 31 in poly(I:C) and flu, 17 in poly(I:C) and IL-6, and 12 genes were similarly changed in all three MIA models. A hierarchical clustering of the expression intensities of these 12 genes clearly separates the saline-treated controls from the flu, poly(I:C) and IL-6 treatments (Fig. 2).

Common gene expression changes among treatments point to involvement of the crystallin gene family

Surprisingly, 5 of the 12 genes that are up-regulated in all 3 MIA treatments belong to the crystallin gene family: crystallin alpha A (*cryaa*), crystallin beta B3 (*crybb3*), crystallin beta B1 (*crybb1*), crystallin beta A1 (*cryba1*) and crystallin gamma B/C (*crygb/crygc*). To validate this finding with an independent method, we generated

cDNA from pooled RNA from embryos in each dam. In order to inquire about a possible effect of location in the uterus, RNA was separately pooled from embryos residing in the left and the right uterine horns, so there were two samples for each dam. For more detailed view on the crystallin family of genes we also tested the expression of crystallin alpha B (*cryab)*, crystallin beta A2 (*cryba2*), crystallin beta A4 (*cryba4*) and crystallin gamma C/A (*crygc/a*). The qPCR data are highly correlated with the microarray data (flu: r=0.82, p=0.007; poly(I:C): r=0.84, p<0.005; rIL-6: r=0.89, p=0.001) (**Fig. 3**). No obvious effect of position is apparent.

Since the expression of these crystallin genes is highly coordinated, we selected only *cryaa*, *cryba1* and *crybb1* for further qPCR analysis using RNA from the individual flu-exposed embryonic brains. The up-regulation of crystallin transcripts observed by microarray is confirmed by the qPCR analysis of the 36 individual embryonic RNA samples. Flu treatment results in significantly increased *cryaa*, *cryba1* and *crybb1* transcript levels (*cryaa:* ddCt = 1.97, p = 0.001; *cryba1*: ddCt = 1.39, p = 0.01; *crybb1*: ddCt = 2.00, p = 0.0002) (**Fig. 4**). However, these data also reveal a remarkable individual variability in flu-induced crystallin up-regulation, which does not correspond to the uterine position of the embryos (flu: SD=2.8; saline: SD=1.6). This indicates that flu exposure does not equally affect all exposed embryonic brains within each dam, which could potentially contribute to eventual behavioral variability.

Crystallin family up-regulation is transient

Since the crystallin family genes are significantly up-regulated at both 3h following IL-6 and poly(I:C) treatment and at 3 days following flu treatment, we asked if

these expression changes persist throughout the lifespan of the offspring. We examined the expression of *cryaa*, *cryba1* and *crybb1* in the frontal cortex and hippocampus of 15 week-old pups (n=12) from poly(I:C)and saline-treated dams. We find no significant difference between these groups in the expression of these genes. Furthermore, no difference is found in the frontal cortex between 6 month-old (n=6) offspring of saline-, rIL-6and poly(I:C)-treated dams. Similarly, in a different cohort of mice, no difference in crystallin expression is seen in the frontal cortex and hippocampus of poly(I:C)and saline-treated dams (n=15) at 12 months of age. Thus, we conclude that the up-regulation of crystallin genes observed across the three MIA models is transient.

Crystallin expression level correlates with placental weight

Maternal immune activation has a strong effect on development, and at least part of this effect is mediated through the influence of maternal IL-6 on the placenta (Hsiao & Patterson, 2011; Mandal, et al., 2010). To test whether there is a relationship between the effects of MIA on the placenta and on the fetal brain, we measured the placental weight of individual embryos from the three MIA conditions (**Fig. 5**). The average placental weight is significantly decreased in all three MIA-treatments compared to saline-treatment (flu: p=0.00016; poly(I:C): p=0.03439; IL-6: p=0.0041). Furthermore, brain expression levels of *cryaa*, *cryba1* and *crybb1* determined by qPCR are highly correlated with the decrease in the average placental weight obtained for the all conditions (*cryaa*: r=0.85, p= 0.072; *cryba1*: r=0.72, p=0.138; *crybb1*: r=0.92, p=0.041). Thus, the response of the crystallin genes corresponds closely with the degree of MIA.

Discussion

This study of the acute effects of MIA on the embryonic brain transcriptome reveals that: 1) all three MIA treatments (flu, poly(I:C) and IL-6) evoke strong gene expression changes in the embryonic brain; 2) the three MIA treatments yield diverse as well as overlapping transcriptome signatures; 3) all MIA treatments strongly up-regulate the crystallin gene family, represented by alpha, beta and gamma members, in the embryonic brain; 4) the up-regulation of crystallin genes is an acute reaction that does not persist into adulthood; 5) the level of crystallin gene expression is correlated with the degree of MIA as measured by placental weight. In addition, our data also reveal a remarkable variability in crystallin induction among the individual brains in the flu MIA model, indicating that this form of MIA does not affect all of the embryos equally within each dam.

While we find that the three MIA models induce a number of genes in common, there are also significant differences among the groups. This can be attributed in part to differences in the molecular action of the three perturbation agents, but also to the differences in the time point of the flu, poly(I:C) and IL-6 exposure. The efficiency of flu exposure and disease time-course are challenging to control, and the flu-triggered cytokine response is gradual and takes days to develop. The time point chosen for IL-6 and poly(I:C) injection (E12.5) approximately mimics the peak of IL-6 induction following flu infection (E9.5) (Shi, et al., 2003; Smith, et al., 2007). However, cytokine induction is longer lasting following infection than it is for the other two treatments. Likewise, the molecular effects of poly(I:C) versus IL-6 at 3 hours after exposure may not be fully equivalent, resulting in both common and divergent gene expression changes

at the same time point. Thus, we believe that the overall data argue that the transcriptome differences we observe are part of the same process, providing molecular "snapshots" of canonical, transient expression changes that are rapidly changing. Nonetheless, the common gene expression patterns across the three MIA models likely give rise to the behavioral deficits that are common in the offspring of all three treatments such as PPI, social interaction, and open field exploration (Shi, et al., 2003; Smith, et al., 2007). This possibility provides strong motivation to further pursue the potential effects of dysregulation of these particular genes on brain development, with a primary focus on the role of the crystallin gene family.

That elevated brain crystallin expression is common among three MIA mouse models aligns well with studies implicating crystallins in human autism and schizophrenia. Altered aB-crystallin protein levels are detected in the frontal cortex of autistic brains (Pickett, 2001). In addition, anti-aB crystallin antibodies are reported to be elevated in sera of autistic subjects compared to controls (Vojdani et al., 2002). Moreover, increased expression of aB-crystallin is detected in Rett syndrome brains (Colantuoni et al., 2001), and aB-crystallin is found in inclusions in brains from patients with Fragile X–associated tremor/ataxia syndrome (Iwahashi et al., 2006). In schizophrenia, altered expression of both a-crystallin and μ-crystallin are reported in the anterior cingulate cortex (Martins-de-Souza et al., 2010) and prefrontal cortex (Arion, Unger, Lewis, Levitt, & Mirnics, 2007; Middleton, Mirnics, Pierri, Lewis, & Levitt, 2002). Furthermore, dysregulated brain crystallin levels are implicated in the manifestation of a variety of neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease (AD), multiple sclerosis, Creutzfeldt-Jakob disease and Alexander

disease (Bajramovic, Lassmann, & van Noort, 1997; Braak, Del Tredici, Sandmann-Kiel, Rub, & Schultz, 2001; Dabir, Trojanowski, Richter-Landsberg, Lee, & Forman, 2004; Lowe et al., 1992; Renkawek, Voorter, Bosman, van Workum, & de Jong, 1994; Shinohara, Inaguma, Goto, Inagaki, & Kato, 1993; Stoevring, Vang, & Christiansen, 2005).

While the exact role of crystallins in these disorders is unclear, studies in animal models suggest that up-regulated crystallin levels serve a neuroprotective function. Knocking out aB-crystallin in AD or experimental autoimmune encephalitis (EAE) mice causes worsened symptoms (Ojha, Karmegam, Masilamoni, Terry, & Cashikar, 2011; Ousman et al., 2007). These effects in EAE mice are in turn ameliorated by systemic delivery of recombinant aB-crystallin (Ousman, et al., 2007), as are the effects of experimental stroke in mice (Arac et al., 2011). Similarly, transgenic over-expression of aB-crystallin ameliorates symptoms in a mouse model of Alexander disease, a fatal developmental neurodegenerative disorder originating in astrocytes (Hagemann, Boelens, Wawrousek, & Messing, 2009). In addition, aA-crystallin exhibits anti-apoptotic protective effects in experimentally-induced inflammation of the uvea (Rao et al., 2008), and pretreatment with a-crystallin blocks systemically induced inflammation in the brain (Masilamoni et al., 2006).

Recent studies have shown that, in addition to their canonical roles as molecular chaperones, crystallins serve a wide variety of other functions that may underlie their protective roles *in vivo*. For example, aA-crystallin-mediated inhibition of caspase-3 rescues Pax6-deficient dopaminergic neurons from apoptosis (Ninkovic et al., 2010). Likewise, aB-crystallin protects against Fas/APO-1-induced cell death *in vitro.*

Crystallins can also confer neuroprotection by modulating cytoplasmic calcium levels. Retinal bB2-crystallin, for example, colocalizes with synaptotagmin 1, a primary calcium sensor, as well as calmodulin, a major calcium-binding protein (Liedtke, Schwamborn, Schroer, & Thanos, 2007a), and several crystallins harbor Greek key motifs that sequester calcium ions (Jobby & Sharma, 2007; Rajini et al., 2001; Sharma et al., 1989). These properties in aand bB2-crystallins are thought to confer their ability to promote neurite-outgrowth during retinal repair (Liedtke, Schwamborn, Schroer, & Thanos, 2007b; Wang, Wang, Wu, Wang, & Yin, 2010). Another pathway of crystallin activity is through regulation of oxidative stress and acute phase immune responses. For instance, aB-crystallin modulates NFκB activity based on its phosphorylation status (Adhikari, Singh, Rao, & Rao Ch, 2011), and pre-treatment with aB-crystallin prevents NFκB-induced neurotoxic effects (Steinman, 2009). Moreover, aB-crystallin adminstration *in vitro* and *in vivo* down-regulates TNFa and nitric oxide synthase in activated microglia (Wu et al., 2009).

Given this literature, the induction of crystallins in response to MIA can be seen as an attempt of the developing brain to counteract the immune system challenge. Importantly, the crystallin up-regulation we observe in embryos in response to MIA cannot be detected in the hippocampus and the frontal cortex of MIA-treated adolescent mice, indicating that crystallin induction is transient and characteristic of the acute phase of the MIA exposure. Furthermore, comparing our data to previous transcriptome studies of MIA offspring suggests that the fetal and adolescent/adult brain transcriptome changes do not share significant commonalities (Asp et al., 2005; Fatemi, Pearce, Brooks, & Sidwell, 2005; Smith, et al., 2007).

In addition to crystallin up-regulation, several other genes are altered in all three models of MIA tested here. For some of them (*mip, si, tnnc2*), there is insufficient knowledge about the function of the proteins they encode, which makes it difficult to envision their particular roles in the brain, while other transcripts are likely to play an important role in brain development. *Aldh1a1* is an important player in environmentally-induced oxidative damage (Lassen et al., 2007), and this might explain its strong up-regulation in these MIA models. As an enzyme, ALDH1A1 exerts a cellular protective role by metabolizing highly reactive aldehydes, including ethanol metabolite acetaldehyde and major products of lipid peroxidation. ALDH1A1 is best known for converting retinaldehyde to retinoic acid, and altered retinoic acid levels in the brain might disrupt normal anteroposterior neural development (Duester, 2008). Similarly, sterol-C4-methyl oxidase-like protein (SC4MOL) is localized to the endoplasmic reticulum membrane and is believed to be involved in cholesterol biosynthesis, which when dysregulated, has clinical manifestations in neurologic development (He et al., 2011). In addition, atonal homolog 7 (*atoh7)* is involved in the development of the optic nerve (N. L. Brown, Dagenais, Chen, & Glaser, 2002). Therefore, the up-regulation of these three genes, *aldh1a1*, *sc4mol* and *atoh7,* may represent an important change in the molecular environment of the embryonic brain that could result in aberrant neural development. Finally, we find that the expression levels of many genes with known roles in cell cycle regulation, neuronal development and differentiation (e.g. cyclin-dependent kinase 12, cyclin-dependent kinase 17, insulin-like growth factor binding protein 3, neural cell adhesion molecule 1 and 2, transforming growth factor beta 2, transducer of

ERBB2, 2, etc.) are highly correlated with the expression of the crystallins (Supplemental Material 2).

The common gene expression pattern across the various MIA treatments, in conjunction with previous findings, argues that IL-6 is a critical mediator of MIA, and that crystallins are important molecular mediators of this process. This raises several important questions. First, is the IL-6/crystallin cascade a promising target for molecular intervention in the context of schizophrenia and autism treatment or prevention? Second, are the currently used anti-inflammatory therapies (which show promise as adjuvant therapy in schizophrenia) modulating the IL-6/crystallin pathway, and acting through this mechanism? Third, how is crystallin overexpression in the embryonic brain disrupting normal development, and does it alter the typical connectivity between the developing brain regions? Finally, what is the critical time window for modulating the IL-6/crystallin pathway during which we can reverse the detrimental behavioral effects of MIA? The answers to these critical questions remain mostly unknown to date, and will have to be answered by comprehensive follow-up experiments.

Overall, based on our findings and the known molecular effects of crystallins we propose that the induction of the crystallin genes in response to MIA is a neuroprotective attempt of the developing brain to counteract environmental stress. However, this response is likely to have detrimental consequences. Due to their additional roles in neuronal differentiation and axonal growth [42, 47] (Liedtke, et al., 2007b), over-expression of crystallins (and other genes such as *aldh1a1*, *sc4mol* and *atoh7*) might tip the delicate balance between neurogenesis and differentiation in the embryonic brain. This view is strongly supported by the observation that poly(I:C) MIA leads to decreased hippocampal

and cortical neurogenesis (De Miranda et al., 2010; Wolf, Melnik, & Kempermann, 2011). We also propose that once the immune activation disappears, the normal brain developmental program resumes, but with a time-lag. As a result, layer formation in the cortex (Soumiya, Fukumitsu, & Furukawa, 2011) and communication between the various brain regions is altered, leading to permanent changes in connectivity and neurochemistry (Deng et al., 2011). This ultimately results in the various behavioral abnormalities that are found in the offspring of all three MIA models. Finally, we also argue that this cascade of events might parallel the mechanisms by which environmental insults contribute to the risk of neurodevelopmental disorders such as schizophrenia and autism.

Methods

Animals and MIA treatments

All procedures involving animals were approved by the Caltech Animal Care and Use Committee. Female C57BL/6J mice (The Jackson Laboratory, Bar Harbor, ME) were obtained from our in-house breeding facility and were housed in ventilated cages under standard laboratory conditions. Mice were mated overnight, and the presence of a vaginal plug marked that day as embryonic day 0 (E0). Pregnant females were not disturbed, except for weekly cage cleaning, until E9.5 when they were weighed and pseudo-randomly assigned to one of four groups. Each group contained four to five pregnant females.

Treatments with human influenza virus, poly(I:C), and recombinant IL-6 (rIL-6) were used to induce maternal immune activation (MIA) at conditions that previously

resulted in offspring altered behavior (Shi, et al., 2003; Smith, et al., 2007). To allow for time to develop a flu infection, pregnant mice on E9.5 were anesthetized intra-peritoneally (i.p.) with 10 mg/kg xylazine and 100 mg/kg ketamine and inoculated intra-nasally with 6000 plaque-forming units of human influenza virus in 90 µl phosphate-buffered saline (PBS). Pregnant mice on E12.5 were injected with saline, poly(I:C) or rIL-6. For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO) was freshly dissolved in saline and administered i.p. at 20 mg/kg based on the weight of the poly(I:C) itself, not including the total weight of the potassium salts. Control mice were injected with saline alone at 5 µl per gram body weight. For rIL-6 injections, 5 µg carrier-free, mouse rIL-6 (eBioscience; San Diego, CA) was freshly dissolved in 150 µl saline and injected i.p. Pregnant mice were sacrificed on E12.5 (3h after poly(I:C) or rIL-6 injection and 3 days after influenza inoculation), and the embryonic brains were removed quickly and frozen in 1.5 ml tubes.

Transcriptome analysis

RNA was isolated from individual embryonic brains using PureLink RNA mini kit (Ambion). RNA quality was assessed using the Agilent (Palo Alto, CA) Bioanalyzer. To reduce gene expression variability that was not related to MIA-exposure, RNA from each embryonic brain within a litter was pooled into a single sample, and used for generation of biotin-labeled sample for microarray hybridization (**Supplemental Material 1**). This allowed us to identify MIA-induced gene expression changes that were commonly found in the embryonic brains of each litter, resulting in 4-5 replicates for each of the 4 conditions. Thus, from a total of 138 embryos, 17 pooled RNA samples

(each originating from all the embryonic brains within an individual pregnant dam) were generated and hybridized on a GeneChip HT MG-430 PM 24-Array Plate. Based on the variability in our previous mouse experiments, we estimated that we had an 80% probability to identify significant gene expression changes that were >30%. The data were log2 transformed using the RMA algorithm, and differential expression was established. A gene was considered differentially expressed if 1) the average log2 ratio (ALR) between the experimental treatment and control exceeded 30% ($|ALR| > 0.3785$) and 2) the statistical significance of differences was $p < 0.05$ using the Student's *t* test. GenePattern software (Broad Institute) was used for hierarchical clustering of the gene expression intensities.

qPCR

RNA isolated from embryos residing in the left or the right uterine horn was pooled proportionately, so there were two samples per each pregnant dam. Individual embryonic brain RNA or pooled RNA samples were used to generate cDNA with High-capacity cDNA reverse transcription kit (Applied Biosystems). SYBR Green intercalation qPCR was run in a 7300 real time PCR system (Applied Biosystems) with the following primers:

cryaa (F: TCTTCTTGGACGTGAAGCAC, R: GAAATGTAGCCATGGTCATCC), *cryab* (F: TGTGAATCTGGACGTGAAGC, R: TGACAGGGATGAAGTGATGG), *cryba1* (F: CCTGGAAAGAGGAGAATACCC, R: TTATGATTAGCGGAACAGATGG), *cryba2* (F: ACCAGCAAAGATGTGGGTTC, R: GCCTAATGCTGGACTCTTCG),

cryba4 (F: CACCACTCAGGTGACTACAAGC, R: CCAGAGGACACAAGGGTAGC), *crybb1* (F: TCCCAGGAACATAAGATCTGC, R: ACGGTCACAGAAGCCATAAAC), *crybb3* (F: CAGCCGACGTAGTGACATTC, R: TCATCTACGATCTCCATCTTGC), *crygb/a/c* (F: AGCGAGATGGGAAAGATCAC, R: AGTACTGGTGGCCCTGGTAG), *crygc/a* (F: TGCGGCTGTATGAGAAAGAA, R: CCTCGGTAGTTAGGCATCTCA). All these custom-designed primers reported amplification efficiency >90%. qPCR data was analyzed using the ddCt method with the housekeeping gene phosphoglycerate kinase 1 (http://www.genenames.org/data/hgnc_data.php?hgnc_id=8896PGK1) as a normalizer.

In addition, qPCR for the crystallin genes *cryaa, cryba1 and crybb1* was performed with 34 RNA samples pooled from the embryos identified as residing in the left or right uterine horns. Furthermore, the expression of *cryaa, cryba1, and crybb1* was also examined by qPCR in all 36 individual embryonic brains originating from the influenza-treated dams.

Correlation of placental weight with gene expression measurements

At the time of the dissection, the weight of the individual placentas was measured for each embryo. Placental weight was assessed across the treatments with a groupwise, two-tailed t-test. In addition, qPCR-reported crystallin expression was correlated with average placental weight across the treatments and control groups using Pearson-correlation.

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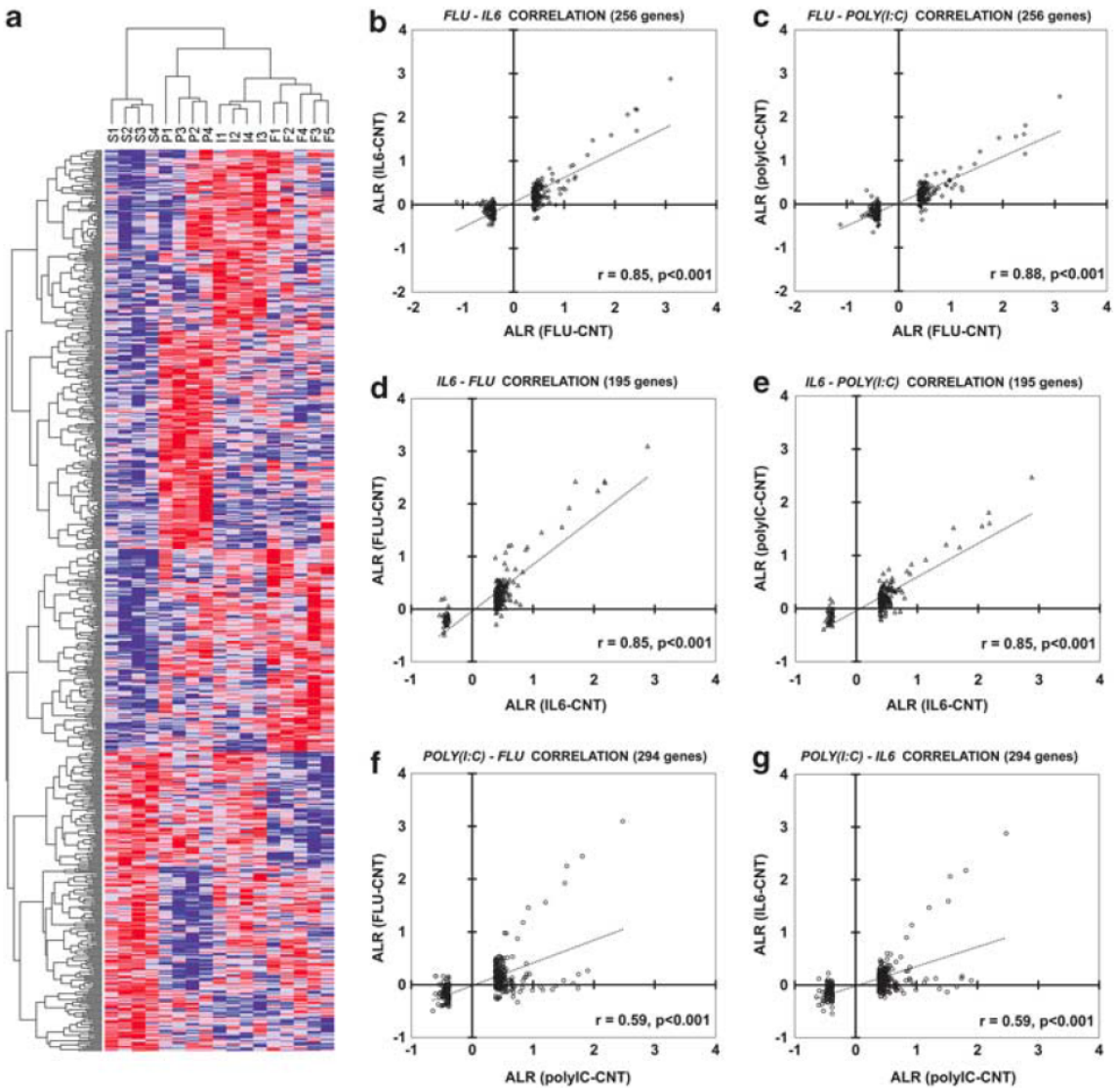

Figure 1. The transcriptome of the embryonic brain is altered in three maternal immune activation (MIA) models. A. All differentially expressed transcripts in the MIA treatments were subjected to a two-way (horizontal:genes; vertical:samples) unsupervised clustering using GenePattern. Gene expression differences are color-coded (red-increased expression; blue-decreased expression), and the intensity of the color corresponds to the magnitude of gene expression change. Note that the experimental groups separate into distinct clusters that correspond to the different MIA treatments, with the main clusters

separating the saline-treatment from all three MIA-treatments. **B-G**. Three dataset-driven gene groups were created, each containing genes that were differentially expressed in one of the three MIA models (256 genes in the flu model, 294 in the poly(I:C) model and 195 in the rIL-6 model). We then examined the expression of each of these gene groups in the other two MIA cohorts. Thus, the pattern of differentially expressed genes in the flu treatment was tested in the poly(I:C) and rIL-6 cohorts (B and C, respectively), the pattern of differentially expressed genes in the rIL-6 treatment was tested in the flu and poly(I:C) cohorts (D and E, respectively), and the pattern of differentially expressed genes in the poly(I:C) treatment was assessed in the flu and rIL-6 cohorts (F and G, respectively). Note that the gene expression patterns uncovered in any of the three MIA treatments show a very strong, highly significant correlation to the other two MIA-treatments.

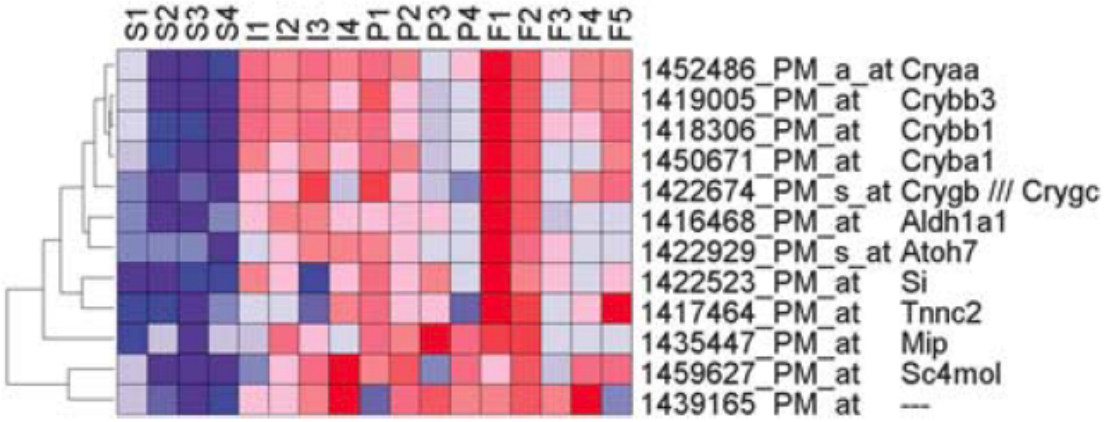

Figure 2. The most significantly changed transcripts in all three MIA treatments include multiple members of the crystallin gene family. Clustering was performed as described in Fig 1A. Note that, of the 12 gene expression changes observed in all three MIA conditions, 5 are mRNA species belonging to the crystallin gene family.

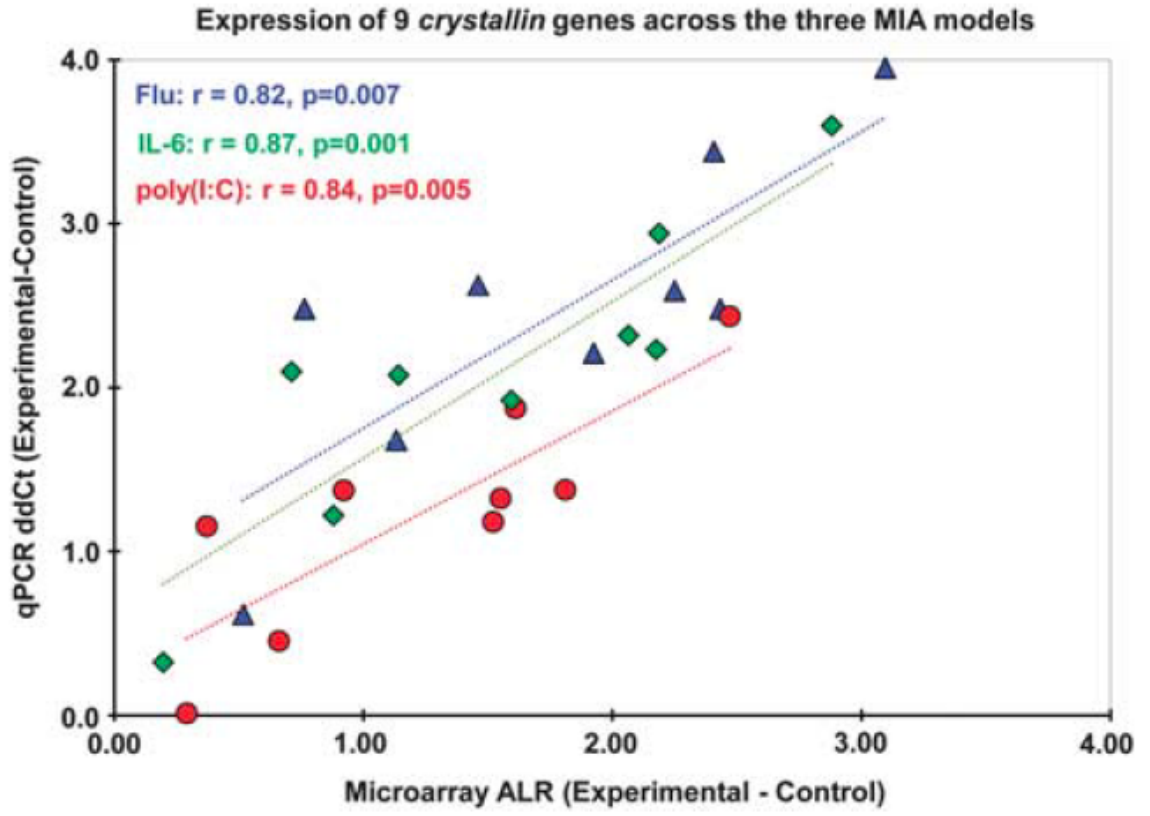

Figure 3. qPCR validates crystallin gene expression changes across the MIA models. The expression of nine genes was tested (Cryaa, Crygb/c/a, Crygc/b/, Crybb1, Cryba2, Crybb3, Cryba1, Cryba4, Cryab) on pools of RNA derived from the three MIA-treatments. The X axis denotes average log2 ratios reported by microarrays, while the ddCt qPCR values are plotted on the Y axis. Note that the qPCR and microarray-reported expression changes are highly correlated across the three MIA models.

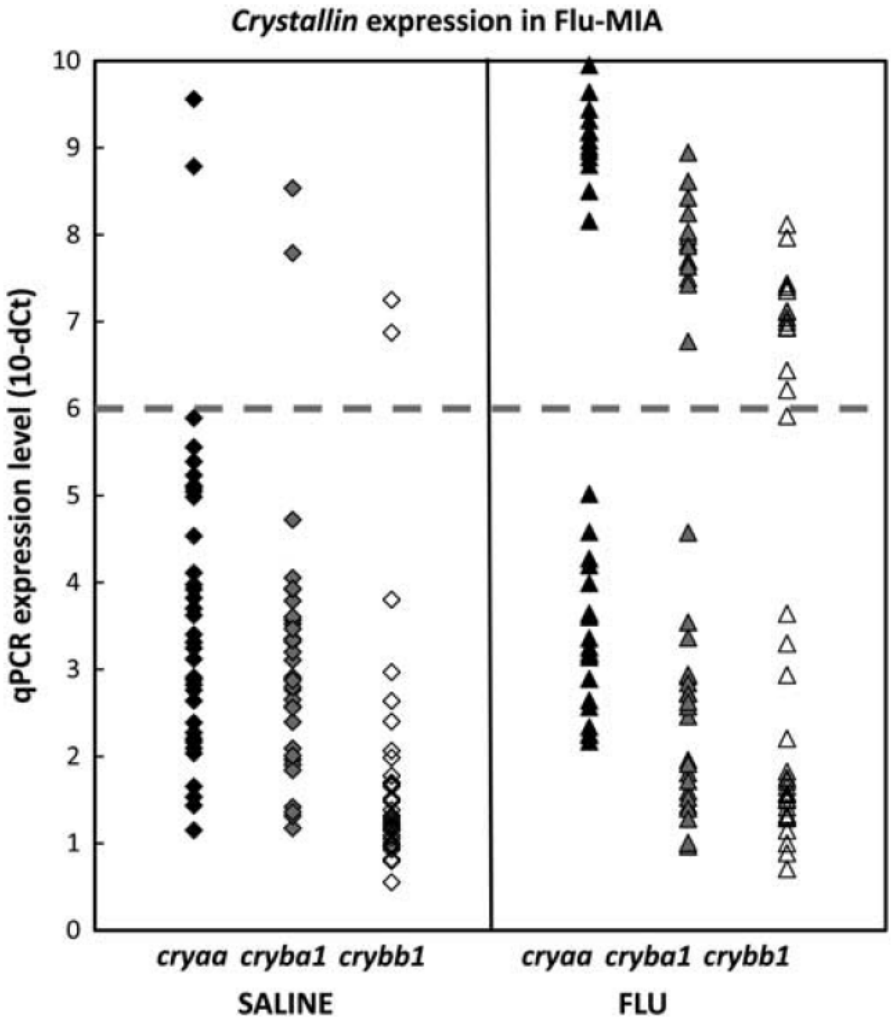

Figure 4. Crystallin expression is variable in individual brains from flu-exposed embryos. Expression of *cryaa*, *cryba1* and *crybb1* was assessed by qPCR across the individual brains of 36 flu and 36 saline embryos. Each symbol represents a PGK1-normalized gene expression level in a single embryonic brain. The dashed gray line denotes the reliable transcript detection threshold by qPCR. Note the up-regulation of the three crystallin genes in the flu group compared to controls, and the considerable variability of crystallin expression levels between the flu-exposed embryonic brains.

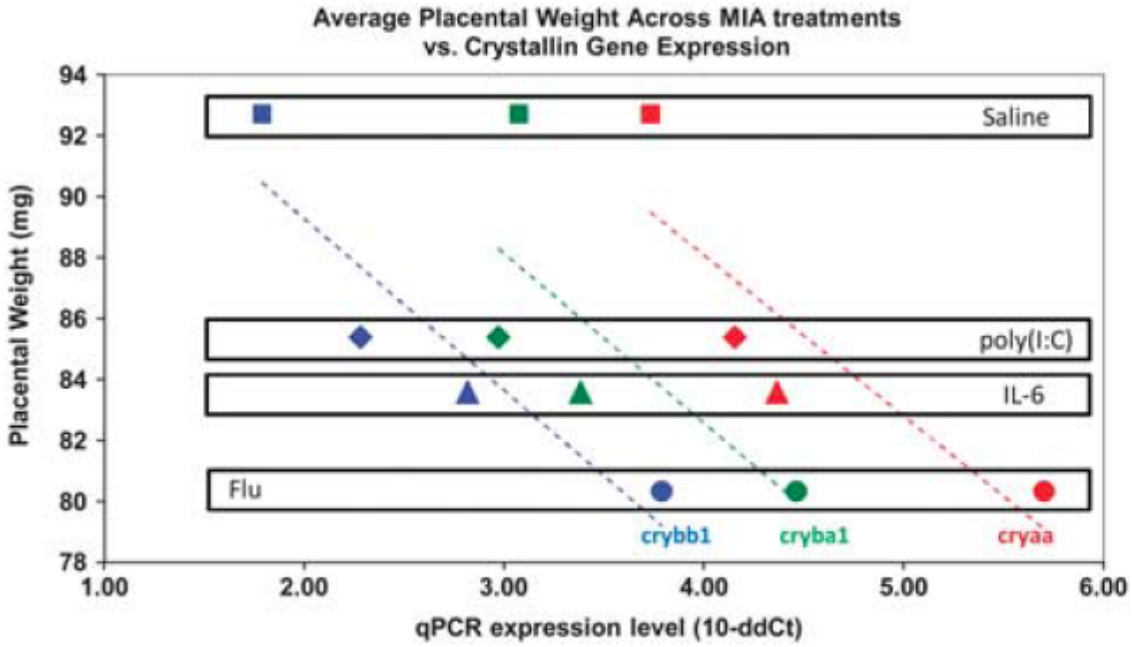

Figure 5. Crystallin expression is correlated with placental weight. qPCR expression levels of *cryaa* (red), *cryba1* (green) and *crybb1* (blue) are correlated with mean placental weights across the MIA groups and controls. The X axis denotes the magnitude of expression change measured in ddCT (1Ct =2 fold change), and the Y axis depicts average placental weight. Squares denote saline treatment, diamonds denote poly(I:C) treatment, triangles denote IL-6 treatment, and circles denote flu treatment. Note that the average placental weight is significantly different between the controls and all three MIA treatments, and that the magnitude of all the crystallin expression changes is highly correlated with placental weight.

	Number of embryos			
Pregnant mouse	Saline	PolyIC	rIL6	Flu
1	10	4	8	8
2	8	12	9	9
3	8	10	6	5
4	10	9	8	7
5	N/A	N/A	N/A	7
Embryos/treatment	36	35	31	36

Supplemental Material 1. Experimental animals and pooling strategy.

The experiment used 17 timed-pregnant mice: 4 controls, 4 poly(I:C)-exposed, 4 IL-6-exposed and 5 flu-exposed. RNA from all embryonic brains within a litter was pooled into a single sample, and used for generation of biotin-labeled samples for microarray hybridization. Thus, from a total of 138 embryos, 17 pooled RNA samples (each originating from all the embryonic brains within a pregnant dam) were generated and hybridized on a GeneChip HT MG-430 PM 24-Array Plate. This allowed us to identify MIA-induced gene expression changes that were commonly found in the embryonic brains of each liter, resulting in 4-5 biological replicates for each of the 4 conditions.

Supplemental Material 2 (Dataset online). Many significant microarray-reported gene expression changes are found in the flu, poly(I:C) and IL-6 MIA models.

Column A: DNA microarray Probe set ID provided by Affymetrix; Columns B-E: RMA-normalized average log2 expression level for the four experimental groups (three MIAand one saline-treated group); Columns F, H, J: Average log2 ratios between the three MIA-treatments and control group. Positive values represent overexpression in experimental groups. Columns G, I, K: Group-wise two-tailed Student t-test values for the three MIA-control comparisons. Column L: Pearson correlation of each gene with *cryaa* expression levels. Column M: NCBI accession ID; Column N: NCBI gene symbol.

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Chapter 8

Maternal immune activation causes age and region-specific changes in brain cytokines in offspring throughout development

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Abstract

Maternal infection is a risk factor for autism spectrum disorder (ASD) and schizophrenia (SZ). Indeed, modeling this risk factor in mice through maternal immune activation (MIA) causes ASD and SZ-like neuropathologies and behaviors in the offspring. Although MIA upregulates pro-inflammatory cytokines in the fetal brain, whether MIA leads to long-lasting changes in brain cytokines during postnatal development remains unknown. Here, we tested this possibility by measuring protein levels of 23 cytokines in the blood and three brain regions from offspring of poly(I:C)and saline-injected mice at five postnatal ages using multiplex arrays. Most cytokines examined are present in sera and brains throughout development. MIA induces changes in the levels of many cytokines in the brains and sera of offspring in a region and age-specific manner. These MIA-induced changes follow a few, unexpected and distinct patterns. In frontal and cingulate cortices, several, mostly pro-inflammatory, cytokines are elevated at birth, followed by decreases during periods of synaptogenesis and plasticity, and increases again in the adult. Cytokines are also altered in postnatal hippocampus, but in a pattern distinct from the other regions. The MIA-induced changes in brain cytokines do not correlate with changes in serum cytokines from the same animals. Finally, these MIA-induced cytokine changes are not accompanied by breaches in the blood-brain barrier, immune cell infiltration or increases in microglial density. Together, these data indicate that MIA leads to long-lasting, region-specific changes in brain cytokines in offspring—similar to those reported for ASD and SZ—that may alter CNS development and behavior.

Introduction

Autism spectrum disorder (ASD) and schizophrenia (SZ) are devastating disorders that each affect cognitive and social functions of approximately 1% of the population (Kogan et al., 2009). Although the etiology of these disorders is unclear, genetics and environmental factors appear to interact to increase risk (Nawa et al., 2000; Patterson, 2009). Indeed, many of the environmental insults linked to SZ and ASD involve the maternal-fetal environment. Large SZ and ASD twin studies highlight the fact that concordance for dizygotic twins is much greater than that for siblings (Brown and Patterson, 2011; Hallmayer et al., 2011; Patterson, 2007; Rosenberg et al., 2009; Szatmari, 2011) and concordance for SZ is also higher for monochorionic twins, who share a placenta, than for dichorionic twins, who do not (Davis et al., 1995). Together, these studies indicate a significant role for the fetal environment in these disorders. In addition, maternal infection greatly increases the risk for SZ and ASD in offspring (Brown and Derkits, 2010; Brown and Patterson, 2011; Patterson, 2011a), and maternal viral infection is associated with increased risk of ASD as well as a 3-7-fold increased risk of SZ in the offspring (Atladottir et al., 2010; Brown et al., 2004; Brown and Patterson, 2011). Since different types of viral, bacterial, and parasitic infections are associated with ASD and SZ, the critical link between prenatal maternal infection and postnatal brain and behavioral pathology appears to be the maternal immune response and factors that mediate that response, such as cytokines (Deverman and Patterson, 2009; Garay and McAllister, 2010).

These correlations from epidemiological studies are supported by work in rodent models of maternal infection. Adult offspring of pregnant mice given intranasal influenza virus exhibit behavioral abnormalities and changes in gene expression, neuroanatomy,

and neurochemistry consistent with both SZ and ASD (Fatemi et al., 2002; Fatemi et al., 1998). Because these outcomes are also elicited in the absence of infection by maternal injection of synthetic dsRNA (poly(I:C)), which mimics the acute phase response to viral infection (Traynor et al., 2004), it is maternal immune activation (MIA) that drives the changes in fetal brain development (Shi et al., 2005). Offspring born to pregnant mice injected with poly(I:C) at embryonic day 12.5 (E12.5) display the three core behavioral symptoms of ASD: stereotyped and repetitive behaviors, deficits in social interaction, and deficits in communication (Malkova et al., 2012; Smith et al., 2007). These offspring also display behaviors that are consistent with both SZ and ASD, including elevated anxiety and deficits in prepulse inhibition (PPI), latent inhibition (LI), and working memory (Patterson, 2011b), some of which can be alleviated by treatment with anti-psychotic drugs (Meyer and Feldon, 2010; Meyer et al., 2010; Piontkewitz et al., 2009; Shi et al., 2003). Adult MIA offspring also exhibit abnormalities in gene expression and neurochemistry similar to those noted in SZ and ASD (Meyer et al., 2011). Finally, neuropathology is also seen in this model, including enlarged ventricles and a spatially-localized deficit in Purkinje cells, characteristic of SZ and ASD, respectively (Meyer et al., 2009; Piontkewitz et al., 2009; Shi et al., 2009). Related findings have also been reported in non-human primate models of maternal infection and poly(I:C) MIA (Bauman, 2011; Short et al., 2010).

Despite recent progress in developing and characterizing rodent MIA models, much remains to be studied about how MIA alters fetal brain development. Current evidence indicates that the maternal cytokine response is crucial (Patterson, 2009), which leads to immune activation and endocrine changes in the placenta (Hsiao and Patterson, 2011;

Mandal et al., 2011). Interleukin (IL)-6 is necessary and sufficient to mediate these effects since the effects of MIA on neuropathology and behavior in the offspring are prevented by injection of pregnant dams with poly(I:C) combined with an anti-IL-6 antibody and are mimicked by a single maternal injection of IL-6 (Smith et al., 2007). Induction of maternal cytokines then alters cytokine expression in the fetal brain, including IL-1β, IL-6, IL-17, IL-13, MCP-1, and MIP1α, hours after MIA (Fatemi et al., 2008; Meyer et al., 2006b; Meyer et al., 2008), with only IL-1β remaining elevated in the fetal brain 24 hours following poly(I:C) injection (Arrode-Bruses and Bruses, 2012). However, it is unknown if MIA causes chronic changes in brain cytokines and/or immune cell infiltration in offspring during postnatal development and/or in adult offspring.

Here, we test this possibility using proteomic analysis of cytokine levels in the postnatal brain. The levels of 23 cytokines were measured in the blood and three brain regions from offspring of poly(I:C)and saline-injected mice at five ages (postnatal day 0 (P0), P7, P14, P30, and P60) using multiplex technology. Perhaps surprisingly, in control brains, most of the 23 cytokines examined are detectable in serum and in all three brain regions (frontal cortex (FC), cingulate cortex (CC), and hippocampus (HC)) from birth through adulthood. The levels of individual cytokines are modulated in age and region-specific patterns that have not been previously described. Most important, MIA induces striking, long-lasting changes in cytokine levels in the brains of offspring, many of which are region and age-specific and include widespread decreases as well as increases, compared to controls. Our results indicate that MIA leads to chronic changes in brain cytokine levels in offspring that may mediate MIA-induced changes in CNS development and behavior.

Results

Several cytokines, including IL-1β, IL-6, IL-10, IFNγ, and TNFα, have been reported to be present in the healthy brain where they perform a wide range of functions (Deverman and Patterson, 2009; Garay and McAllister, 2010). However, it was unknown if other cytokines and chemokines are present in early postnatal development in the healthy brain, and if so, whether their levels change with age and following MIA. Here, we utilized the approach previously published for 3-plex BioRad luminex kits (Datta and Opp, 2008) for use with BioRad luminex mouse 23-plex bioplex kits. The high sensitivity of this approach allowed us to measure levels of 23 cytokines and chemokines from small regions of the developing mouse brain. The cytokines measured were IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-17, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), granulocyte colony-stimulating factor (G-CSF), and granulocyte-macrophage colony-stimulating factor (GM-CSF). The chemokines measured included Eotaxin, keratinocyte chemoattractant (KC/CXCL1), monocyte chemoattractant protein-1 (MCP-1/CCL2), macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP1β/CCL4, and regulated upon activation, normal T-cell expressed, and secreted (RANTES/CCL5).

Levels of these factors were measured in FC, CC, and HC at P0, P7, P14, P30, and P60. These brain regions were selected based on their involvement in ASD and SZ. The five ages represent periods before most synapses have formed (P0), the start of synaptogenesis (P7), the peak of synaptogenesis (P14), peak activity-dependent plasticity (P30), and early adulthood (P60). An average of six animals from at least two litters were

used for each data point. Samples were run in duplicate and replicated on at least two arrays. Litters were obtained from pregnant mice injected at mid-gestation either with saline (typically-developing or control offspring) or with 20 mg/kg poly(I:C). We use maternal polyI:C injection at E12.5, since this stage of gestation correlates with the late first trimester in humans (Clancy et al 2007)—the time that infections are most closely linked to increased incidence of SZ and ASD (Atladottir et al. 2010, Brown et al. 2004).

Most cytokines are expressed in an age and region-specific manner in the brain and blood of typically-developing offspring from birth to adulthood

Surprisingly, most of the cytokines and chemokines examined are detectable in both brain and serum from control offspring, even at birth (Fig. 1). Although a few of the cytokines are undetectable in specific brain regions or serum during the first postnatal week, including IL-1α, IL-3, IL-4, IL-5, Eotaxin, G-CSF, and RANTES, all are detectable in brain and serum after the first postnatal week (Suppl. Tables 1-2). Most of the cytokine concentrations range from 1-100 pg/mg brain tissue. Cytokines with the lowest concentrations in multiple brain regions include IL-1α, TNFα, IL-3, IL-4, IL-5, G-CSF, and RANTES. Conversely, cytokines with the highest concentrations in multiple brain regions include IL-9, IL-13, and Eotaxin. Almost all cytokines are found at higher concentrations in serum (Fig. 1 M-P) compared to brain (Fig. 1 A-L; note that the scale is 10-fold greater for serum compared to brain regions).

Cytokine concentrations in the typically-developing brain change with age and some changes are region-specific. To illustrate changes in cytokine concentrations with age, the average values for each cytokine concentration (pg/mg from brain and pg/ml

from serum) are plotted at the five ages examined (Fig. 1). Error bars are not included in these graphs to enhance visibility of the trends in expression of each cytokine with age, however all values of mean ± SEM concentration are included in Suppl. Tables 1-2. For presentation, we separated the 23 cytokines into four groups: (1) commonly-studied cytokines, (2) a first set of additional cytokines with a similar step-like increase in serum concentration with age, (3) a second set of additional cytokines with stable levels in serum, and (4) chemokines.

Although the developmental profiles of these cytokines are diverse, some generalizations can be made. First, most cytokine profiles are similar across the three brain areas. This is most clearly seen for the chemokines, where the profiles for each chemokine in the three brain areas are nearly identical (Fig. 1D, H, L). There are, however, clear exceptions to this generalization, particularly in FC. For instance, IL-1β levels in HC and CC are similar with age, but quite different in FC where they are highest at P0 and P7 and then dramatically decrease at P14 (Fig. 1A, E, I). Levels of IL-9 are steadily high with age in CC and HC and are also relatively high in FC during the period of rapid synaptogenesis (P0-P14), but decrease in FC with maturity (Fig. 1C, G, K). Conversely, IL-6 decreases at P60 in HC and CC, but not in FC where it remains high in adulthood (Fig. 1A, E, I). A second point of interest is that some cytokines, including IL-4, IL-2, IL-17, are higher in mid-postnatal life, but lower at birth and in the adult (Fig. 1B-C, F-G, J-K). Third, several cytokines dip in concentration specifically at P14, a period of intense synaptogenesis; these include IL-3, IL-13, IL-12 (p40), Eotaxin, MIP1α, and KC in addition to IL-2 and IL-5 specifically in CC and HC (Fig. 1A-L). Fourth, another set of cytokines increase in concentration with age, including IL-6, IL-10,

IFNγ, GM-CSF, IL-12 (p70), IL-17, and MIP1β (Fig. 1A-L). Finally, the concentration of a subgroup of cytokines does not change with age, including TNFα, G-CSF, MCP-1, and RANTES, in addition to IL-1α in FC and IL-10, IL-5, and GM-CSF in HC (Fig.1A-L). Together, these results indicate that cytokines are expressed in the typically developing brain in an ageand region-specific manner.

Cytokine concentrations also change with age in serum (Fig. 1M-P), but the patterns are simpler than in brain and fall into just two distinct groups. Regardless of their starting concentration, 17 of the 23 cytokine levels are steady from P0 to P7 and then increase abruptly to a higher level at P14, where they remain through P60 (Fig. 1M-N,P). Importantly, this trajectory is not found in any area of the brain, indicating that the brain measurements are not significantly contaminated by blood cytokines and that changes in peripheral cytokines do not reflect the direction of cytokine changes in the CNS. The six other cytokines in the serum (TNFa, IL-2, -5, -9, -12, -17) remain at a constant level throughout development, a pattern that is also rare in the brain (Fig. 1O).

MIA alters brain cytokine profiles in an age-and region-specific manner

In order to determine if brain cytokines are altered in newborn MIA offspring and if these changes are developmentally regulated, pregnant mice were injected with poly(I:C) (MIA) or saline (control) in mid-gestation (E12.5) and FC, CC, and HC were collected from the offspring at P0, P7, P14, P30, and P60. The protein levels of 23 cytokines were measured as above on the same plates as the age-matched control samples. Average values were compared between saline and poly(I:C) offspring for each cytokine at each age in each brain region. Previous reports using ELISAs to measure

cytokines in fetal brain of MIA offspring showed that some pro-inflammatory cytokines (IL-1β, IL-6, TNFα, and IFNγ) are increased in the brains of offspring during gestation within hours following MIA (Fatemi et al., 2008; Meyer et al., 2006b; Meyer et al., 2008). However, those studies did not determine if brain cytokines are chronically altered in postnatal MIA offspring and if so, what the trajectory of those changes is with age.

Frontal Cortex (FC)

In FC, cytokine levels are altered at every age examined in MIA offspring (Figure 2A-F, Suppl. Table 1) and follow a clear pattern of elevations at birth, decreases throughout postnatal brain development (P7-P30), and elevations again in the adult. Compared to controls, many cytokines in MIA FC are higher at birth and in adulthood. At birth (Fig. 2A, F), four cytokines are significantly higher than controls: IL-1β (1.9-fold), IL-10 (2.8-fold), IL-12 (p70; 2.2-fold), and GM-CSF (3.1-fold). Although not statistically significant, IL-6 is dramatically higher (3.3-fold, p=0.09), and IL-1α (1.4-fold; p=0.07) and IL-4 (1.3-fold; p=0.09) trend toward higher levels. A somewhat different subset of cytokines is also higher in the adult FC (Fig. 2E-F), including IL-1α (2.5-fold), IL-6 (1.7-fold), IL-9 (1.4-fold), and IL-10 (1.3-fold).

In contrast to the increased levels in MIA FC cytokines at birth and in the adult, many cytokines are lower than controls during the period of synaptogenesis and remodeling (P7, P14, and P30). Most of the cytokines that are elevated at P0 are not altered at P7 except for IL-10, which changes from being elevated 2.8-fold at P0 to being decreased (0.8-fold) at P7 (Fig. 2B,F). Including IL-10, six cytokines are significantly altered at P7. IL-2 (0.7-fold), IL-4 (0.3-fold), IL-5 (0.4-fold), and IL-12 (p40; 0.5-fold) are lower, while

G-CSF is the lone cytokine that is higher at P7 (1.5-fold). Several additional cytokines trend toward significantly lower levels than controls, including IL-3 (0.2-fold, p=0.08) and IFNγ (0.4-fold, p=0.09). At P14 (Fig. 2C,F), several of these cytokines remain lower, including IL-2 (0.6-fold), IL-5 (0.5-fold), and IL-10 (0.7-fold) and an additional eight cytokines are lower at this age, including IL-1α (0.4-fold), IL-1β (0.6-fold), IL-9 (0.8-fold), IL-13 (0.8-fold), Eotaxin (0.7-fold), GM-CSF (0.8-fold), IFNγ (0.8-fold), and MCP-1 (0.7-fold). At P14, there are also trends toward significantly lower levels than controls (MIP-1β (0.8-fold, p=0.07) and IL-3 (0.7-fold, p=0.06)). Many cytokines remain lower than controls at P30 (Fig. 2D,F) including IL-1β (0.8-fold), IL-5 (0.6-fold), IL-10 (0.8-fold), GM-CSF (0.6-fold), and MCP-1 (0.7-fold), and an additional six cytokines are also lower at this age, including IL-3 (0.7-fold), IL-6 (0.6-fold), IL-12 (p40; 0.8-fold), IL-12 (p70; 0.8-fold), G-CSF (0.5-fold), and MIP-1β (0.7-fold).

Together, these results indicate that cytokines are indeed chronically altered in the FC of postnatal MIA offspring in a distinct developmental pattern. Both the subsets of cytokines that are altered and the direction of change are age-dependent. Perhaps most surprising, many cytokines are altered in the MIA FC and the majority of these cytokines are significantly decreased during the periods of circuit formation and plasticity within the FC (Fig. 2F).

Cingulate Cortex (CC)

In CC, cytokine levels are also changed at every age examined in MIA offspring compared to controls (Figure 3A-F, Suppl. Table 1) in a pattern similar to that found in FC. As in FC, cytokines are generally higher at birth and in adulthood and lower during

the periods of synaptogenesis and plasticity (P7, P14, and P30). At birth (Fig. 3A,F), three cytokines are significantly higher than controls: IFNγ (1.4-fold), IL-12 (p70; 1.6-fold), and MCP-1 (1.4-fold). Although not statistically significant, IL-1α (1.9-fold, p=0.07) and IL-10 (1.7-fold, p=0.06) are also higher in the CC at birth. A somewhat different subset of cytokines is higher in the adult CC (Fig. 3E,F), including IL-10 (1.4-fold) and IFNγ (1.5-fold). MIP1α (1.45-fold) and RANTES (1.6-fold) are also close to significance and TNFα and IL-2 are dramatically higher (3.4 and 2.9-fold, respectively) but not statistically significant in the adult MIA CC.

Similar to the pattern in FC, many cytokines are lower than controls during the periods of synaptogenesis and remodeling. By P7 (Fig. 3B,F), all of the cytokines that are higher at P0 return to control levels, but a new set of six cytokines is significantly altered: five are lower, including IL-2 (0.4-fold), IL-5 (0.6-fold), IL-6 (0.3-fold), IL-10 (0.7-fold), and Eotaxin (0.6-fold), while IL-17 is dramatically higher (2.2-fold). Although not statistically significant, IFNγ (0.3-fold, p=0.08), IL-12 (p70; 0.7-fold, p=0.07), and KC (1.5-fold, p=0.06) also trend away from controls in MIA CC. Of the cytokines altered at P7, IL-10 remains lower at P14 (0.7-fold) as does IL-17, but the direction of change in IL-17 is reversed (0.8-fold; Fig. 3C,F). In addition, five different cytokines are significantly lower in the P14 MIA CC, including IL-1β (0.6-fold), GM-CSF (0.63-fold), IFNγ (0.7-fold), KC (0.7-fold), MCP-1 (0.6-fold), and MIP-1β (0.7-fold). Although Eotaxin and MIP-1α are also lower in P14 MIA CC (Fig. 3C), they are not statistically different from controls. At P30, even more cytokines are lower in MIA CC compared to controls (Fig. 3D,F). Four cytokines that are lower at P14 remain lower than control levels at P30, including IL-1β (0.8-fold), IL-10 (0.7-fold), IL-17 (0.7-fold), and MCP-1

(0.8-fold) and seven additional cytokines are also lower at P30: IL-3 (0.7-fold), IL-4 (0.7-fold), IL-5 (0.7-fold), IL-6 (0.2-fold), IL-12 (p40; 0.8-fold), IL-12 (p70; 0.8-fold), and G-CSF (0.6-fold). IL-13 (0.7-fold, p=0.08), IFNγ (0.7-fold, p=0.08), and MIP1α (0.8-fold, p=0.08) are also close to significantly decreased at P30.

Hippocampus (HC)

In HC, MIA induces changes in the levels of cytokine at every age examined except for P60 (Fig. 4A-F, Suppl. Table 2) in a pattern distinct from that found in FC and CC. However, unlike the clear directional switch in MIA-induced changes in FC and CC in the first postnatal week, the direction of change in cytokine levels is mixed at most ages in HC. At birth (Fig. 4A,F), seven cytokines are altered in MIA HC. IL-6 (1.6-fold) is significantly elevated over controls, while IL-1β (0.7-fold), IL-2 (0.6-fold), IL-4 (0.8-fold), KC (0.7-fold), MCP-1 (0.7-fold), and MIP-1α (0.3-fold) are lower. Although not statistically significant, IL-13 is also lower at birth (0.7-fold, p=0.06). Of the cytokines altered at P0, only IL-4, KC, and MIP-1α remain changed at P7 (Fig. 4B,F): IL-4 remains lower (0.6-fold) and KC and MIP1α reverse direction towards a large elevation at P7 (1.6-fold; 1.6-fold). Four different cytokines are altered at P7 including IL-3 (0.4-fold), IL-5 (0.7-fold), IL-9 (1.6-fold), and IL-10 (0.8-fold). At P14, eight cytokines are significantly different in MIA HC compared to controls (Fig. 4C,F). Compared to the cytokines altered at P7, only IL-5 (0.4-fold) remains lower, and IL-9 is reversed in direction from being higher at P7 to being lower at P14 (0.7-fold). At P14, six different cytokines are altered in MIA HC: IL-1α (1.4-fold), IL-2 (0.4-fold), IL-6 (1.5-fold), Eotaxin (0.7-fold), MCP-1 (0.8-fold), and RANTES (0.6-fold). At this age, IL-10, IL-12

(p40), and MIP-1β are also lower than controls, although not significantly. Far fewer cytokines are altered in MIA HC at P30 (Fig. 4D,F). Only two cytokines are significantly lower than controls—IL-6 (0.6-fold) and MIP-1α (0.8-fold)—although several other cytokine changes approach significance including IL-1β (0.8-fold; p=0.09), IL-12 (p70; 0.7-fold; p=0.06), IL-13 (0.7-fold, p=0.08), G-CSF (0.6-fold, p=0.08), and RANTES (0.8-fold, p=0.09). Compared to controls, no cytokines are significantly altered in HC in adult MIA offspring (Fig. 4E, F).

MIA alters cytokine profiles in serum in an age-specific manner

MIA also causes chronic changes in cytokine levels in the serum of offspring throughout postnatal development (Figure 5A-F, Suppl. Table 2). At birth, five cytokines are altered in MIA serum compared to controls. IL-12 (p40; 1.8-fold) and RANTES (2.4-fold) are higher, while IL-3 (0.7-fold), GM-CSF (0.8-fold), and MIPα (0.5-fold) are lower than controls (Fig. 5A,F). However, the most dramatic changes are one week after birth (P7), when twelve cytokines are altered in MIA serum (Fig. 5B,F). IL-1β (1.9-fold), IL-3 (1.4-fold), IL-6 (1.5-fold), IL-12 (p40; 3.0-fold), G-CSF (2.6-fold), IFNγ (1.3-fold), KC (2.2-fold), RANTES (1.9-fold), and TNFα (1.5-fold) are all higher, while IL-1α (0.6-fold), IL-2 (0.7-fold), and IL-12 (p70; 0.6-fold) are lower relative to controls at P7. By P14 (Fig. 5C,F), most of these widespread changes in MIA sera are back to control levels except for TNFα, which remains slightly higher (1.2-fold) and MIP-1β, which is significantly higher (1.1-fold). Although not statistically significant, IL-10 is also higher at P14 (1.5-fold, p=0.07). At P30, the cytokine profile is changed again, and four different cytokines are altered: IL-1β (1.7-fold), IL-6 (1.4-fold), and IL-9 (1.2-fold) are

higher than controls, while IL-3 (0.6-fold) is lower (Fig, 5D,F). In adults, no significant differences in cytokine levels relative to controls are found in sera of MIA offspring (P60; Fig. 5E-F).

MIA-induced changes in serum cytokines do not correlate with changes in brain cytokines

As illustrated in the summary Fig. 6, MIA causes changes in cytokine levels in brain and serum in offspring throughout postnatal development. In this figure, only statistically significant changes in cytokine levels are indicated by colored boxes; the direction and magnitude of change is indicated by red for higher, and blue for lower, levels than controls. These MIA-induced changes are complex, being both age and region-specific, and involve significant changes at some point during development in levels of 22 of the 23 cytokines assayed in brain (the exception being TNFα). In striking contrast to the generally consistent cytokine profiles between brain regions in the control brain, at a given age, the nature and direction of MIA-induced changes in cytokines are different between brain regions. Moreover, within each brain region, the MIA-induced changes are different between ages. Although the changes are complex, they are not random; instead, they fall into a few, distinct patterns. In general, compared to controls, many cytokines in FC and CC are higher at P0, lower from P7-P30, and then higher again in the adult, although the specific cytokines that are altered at each age differ between these two regions. This general pattern of cytokine change is distinct from that in the HC, where fewer cytokines are altered and their direction of change is mixed at each age. Finally, the changes in cytokine levels observed in serum are also different at each age,

but as in the control brains, these changes do not correlate with changes in cytokine levels in any brain region from the same animals. This supports the conclusion that our brain cytokine measurements are unlikely to be contaminated by serum cytokines. Finally, although cytokine levels are altered in all three brain regions and serum throughout postnatal development, they remain altered in the adult only in FC and CC, but not in HC or serum.

MIA-induced changes in brain cytokines are not due to immune cell infiltration through a leaky blood-brain barrier

Changes in brain cytokines in MIA offspring could be due to cytokines produced by infiltrating immune cells, which might gain access to the brain through MIA-induced breaches in the blood-brain barrier (BBB). To test this possibility, first BBB integrity was qualitatively assessed using Evans Blue (Suppl. Fig. 1); breaches in BBB integrity are typically indicated by dark blue staining of the brain. There is little to no BBB permeability detected at any postnatal age examined and no change in staining of MIA brains compared to controls at any age tested. Second, immune cell infiltration into the three brain regions was examined using immunohistochemistry with cell-type-specific antibodies to label B and T lymphocytes and neutrophils (Suppl. Fig. 2A). In general, there is virtually no staining for any of these immune cell types in any region of the brain at any age examined. This lack of staining is not due to an inability of our protocol to detect these cells, since we obtain clear labeling of these cells in spleens of control mice and spinal cord from EAE mice (Suppl. Fig. 2B). Taken together, these results support

the interpretation that the MIA-induced changes in brain cytokines are most likely produced by brain-resident cells.

Microglial number is not altered by MIA

Microglia are present in the brain during all stages of development and are well-documented to increase in number and state of activation during neural inflammation (David and Kroner, 2011; Ransohoff and Perry, 2009). Because of the subjectivity inherent in classifying microglial activation morphologically, we chose to quantify only microglial density in sections from MIA and control brains. Microglia were labeled with the standard marker, Iba1, using immunohistochemistry and counted using Stereologer (Fig. 7). There are no qualitative changes in microglial morphology in any brain region from MIA offspring compared to controls (Fig. 7A). In general, the density of microglia in each brain region increases from low levels at birth to a peak at P14 and then decreases to intermediate levels in the adult brain (Fig. 7B). MIA does not cause any significant change in microglial density within any of the three brain regions at any of the five ages examined (Fig. 7B).

Behavioral abnormalities in MIA offspring

Previous studies have documented ASD and SZ-relevant abnormal behaviors in adult poly(I:C)-induced MIA offspring (Meyer and Feldon, 2010). To confirm that the MIA mice used in this study were likely to exhibit the expected behavioral changes, we tested two of these behaviors in a parallel cohort of animals that was raised alongside the cohort used for cytokine measurements. The two behavioral tests performed were LI and

PPI. LI is a measure of the ability to ignore irrelevant stimuli and its disruption is considered to be pertinent for the cognitive deficits in SZ (Weiner, 2003). LI is disrupted in SZ subjects and in amphetamine-treated humans and rats, restored to normal levels in SZ by neuroleptic drugs, and enhanced in normal humans and rats by antipsychotic drugs (Weiner, 2003). PPI measures sensorimotor gating, and deficits are present in ASD, SZ and several other mental disorders. As in prior work (Meyer et al., 2009; Patterson, 2009) we find deficits in LI and PPI in the parallel cohort of mice used here (data not shown). MIA offspring exhibit increased freezing in response to a conditioned acoustic cue (LI) compared to controls when measured against the non-pre-exposed group [$F(1, 23)=1.029$, $p=0.0149$; n=28 saline, 24 poly(I:C)]. Compared to control offspring, MIA offspring also display decreased PPI when presented with either a 5 dB pre-pulse or 15 dB pre-pulse preceding a 120 dB pulse (PPI5 and PPI15, respectively) [$F(1, 104)=4.830$, $p=0.0302$; n=29 saline, 25 poly(I:C)].

Discussion

In order to determine if poly(I:C)-induced MIA leads to chronic changes in brain and blood cytokines in offspring throughout postnatal development, proteomic analysis of the cytokine response in the developing brains of offspring was performed. In typically-developing, control offspring, we find that most of the cytokines examined are present in serum as well as in the three brain regions examined from birth through adulthood. This data adds new evidence to the case that cytokines play a role in normal brain development and function (Deverman and Patterson, 2009; Garay and McAllister, 2010; Gilmore et al., 2004; Juttler et al., 2002; McAfoose and Baune, 2009; Stellwagen, 2011).

Another striking observation from the control data is that the levels of 17 of the 23 cytokines assayed in serum increase abruptly on P14, and they remain at that level through P60. Although many aspects of immune system development occur during the second postnatal week in mice, the precise events that lead to this abrupt increase in serum cytokines are unknown. Importantly, this jump in cytokine levels is not found for any of the 23 cytokines in any of the brain areas, indicating that the measurements in the brain are not significantly contaminated by serum cytokines.

Remarkably, most of the 23 cytokines examined are altered in the brains of MIA offspring and these changes occur in a regionand age-specific manner. While several of the commonly studied, pro-inflammatory cytokines are altered by MIA, significant and widespread changes in anti-inflammatory cytokines and chemokines are also detected in brain and serum at every age examined. Although complex, MIA-induced changes in brain cytokines fall into a few, distinct patterns. In FC and CC, many proand anti-inflammatory cytokines are elevated in early postnatal MIA offspring, lower than controls in early adolescence, and a few cytokines are again elevated in the adult brain. The developmental pattern in HC is distinct from the other brain areas, with both increases and decreases in cytokines occurring at every age examined, except in the adult. Finally, these striking and complex changes in brain and serum cytokines are not accompanied by obvious changes in BBB permeability, immune cell infiltration, or increases in microglial density. Together, these data indicate that MIA leads to significant changes in brain cytokines in the postnatal offspring, which may alter CNS development and behavior in the absence of overt signs of neural inflammation.

The poly(I:C) MIA mouse model employed in this study has both face and construct validity for ASD and SZ, and predictive validity for at least SZ (Meyer and Feldon, 2010). Despite recent progress in developing and characterizing rodent MIA models, little is known about how MIA alters fetal brain development. Current evidence indicates that the maternal cytokine response is crucial (Patterson, 2009). Poly(I:C) injection at either midor late gestation leads to marked increases in levels of IL-1β, IL-6, IL-10, and TNF-α in the pregnant dam's serum (Gilmore et al., 2005; Koga et al., 2009; Meyer et al., 2006b), which is reminiscent of similar increases in serum cytokines in mothers of children with ASD (Goines et al., 2011). Perturbation experiments indicate that IL-6 is necessary and sufficient to mediate the effects of the maternal immune response in the fetus (Smith et al., 2007). Induction of maternal cytokines then alters cytokine expression in the fetal brain, including IL-1β, -6, -10, -13, and -17, and MCP-1 and MIP1α, hours after MIA (Arrode-Bruses and Bruses, 2012; Meyer et al., 2006b), but it has been unclear if MIA causes changes in brain cytokines in postnatal offspring.

It has been suggested that brain cytokines would be altered in MIA offspring based on observations of neural inflammation and cytokine changes in blood, CSF, and postmortem brain tissue from individuals with ASD and SZ (Ashwood et al., 2011; Ashwood et al., 2011; Ashwood et al., 2010; Chez et al., 2007; Molloy et al., 2006; Pardo et al., 2005; Vargas et al., 2005). These human data predict that pro-inflammatory cytokines may be chronically increased in MIA brains and indeed, most of the few cytokines previously detected in the brains of MIA offspring were acute phase cytokines including IL-6, IL-1β, IFNγ, and TNFα (Gilmore et al., 2005; et al., 2006b; Meyer et al., 2008). Here, we took an unbiased approach to test this hypothesis and measured cytokine

levels throughout postnatal development. Our results clearly demonstrate that MIA leads to dynamic changes in cytokine levels in the brains of offspring. Our results are also somewhat consistent with the expected pro-inflammatory changes in brain cytokines resulting from MIA in that there are increases in some of the acute phase cytokines at birth and in the adult MIA FC, CC, and HC, as well as in serum throughout development.

Although it is theoretically possible that these MIA-induced changes in brain cytokines in offspring could be influenced by changes in the dam's behavior following MIA, there is little evidence supporting this possibility. The dosage of poly(I:C) administered results in an approximately three hour period of sickness behavior (lethargy, lack of grooming, etc.), after which the mother recovers fully. Consistent with this, levels of maternal and placental pro-inflammatory cytokines peak by 3 hours post injection and decline considerably by 24 hours post injection (Meyer et al. 2006). The dam loses weight (~1.0-1.5 g) by 24 hours post-injection, but recovers to levels comparable to controls by 48 hours post injection. We have found no difference between poly (I:C) and saline-injected mothers in maternal care, as measured by equivalent time spent licking/nursing pups and comparable latency to retrieve individual pups removed from the nest (Malkova and Patterson, personal communication). Although cross-fostering control offspring with immune-challenged mothers can alter behavior in control offspring (Meyer et al. 2006c), there is extensive evidence that cross-fostering MIA offspring with control mothers does not protect against the emergence of neuropathology and behaviors in the offspring (Meyer et al. 2008). Together, current data in the field is most consistent with the hypothesis that MIA causes changes in brain development that lead to ASDand SZ-like behaviors in offspring.

Despite evidence for a pro-inflammatory brain cytokine profile at birth and in the adult MIA offspring, our results also reveal that MIA induces long-lasting changes in a surprisingly wide range of cytokines. These cytokines include both pro-inflammatory, anti-inflammatory, and regulatory cytokines, as well as several chemokines. In fact, most of the cytokines examined are significantly altered at some point in the MIA brain compared to controls. Perhaps most important, our results also reveal an unexpected and widespread *decrease* relative to controls in many brain cytokines during peak periods of synaptogenesis and plasticity (P7-P30). These decreases are in contrast to the expected pro-inflammatory phenotype and suggest that dramatically decreased cytokine signaling may be a critical variable causing altered brain connectivity and ASD and SZ-like behaviors in the offspring. Consistent with this idea, decreases in a few cytokines have been reported in postmortem brain tissue from individuals with ASD (Vargas et al., 2005) and SZ (Toyooka et al., 2003). The impact of widespread decreases in brain cytokines on the development and plasticity of cortical connections and synaptic transmission and plasticity will be the focus of future studies.

There is a rich complexity in the regionand age-specific changes in MIA brain cytokines. There are only a few reports of brain region-specific changes in cytokine levels following any manipulation, but our data and the few reports in non-MIA mouse models (Johnson and Kan, 2010; Kasten-Jolly et al., 2011) are consistent with the single report of region-specific changes in postmortem brain tissue from individuals with ASD (Vargas et al., 2005). Region-specific changes in overall gene expression have also been reported for SZ and ASD (Focking et al., 2011; Ji et al., 2011; Suda et al., 2011), suggesting that regional-specificity may contribute to both disorders. Detection of region-

specificity of MIA-induced cytokine changes during development was made possible only recently due to the increased sensitivity of multiplex analysis from small amounts of tissue (Datta and Opp, 2008). The advantage of the Luminex x-map technology over traditional ELISAs is that the former requires only a small sample volume (50µl) to simultaneously measure multiple analytes. Luminex technology is being utilized to measure adult brain cytokines in an increasing number of reports (Abazyan et al., 2010; Dalgard et al., 2012; Datta and Opp, 2008; Erickson and Banks, 2011; Gandhi et al., 2007; Li et al., 2009; Mukherjee et al., 2011), and the greater sensitivity of the Bioplex system we used has been validated against both ELISA and other multi-plex systems (Fu et al., 2010; Mukherjee et al., 2011). Our results clearly demonstrate that a single, common maternal immune response (MIA during mid-gestation) can cause long-lasting changes in cytokines that are specific to particular brain regions. How this occurs is unknown, but may involve differential responses to MIA-induced cytokines elevated in fetal development mediated by well-documented region-specific differences in the distribution of cytokine receptors in the normal fetal brain (Bauer et al., 2007; Garay and McAllister, 2010). In the future, additional research is needed to determine if these region-specific patterns of changes in brain cytokines contribute to specific ASDand SZ-like behaviors in offspring.

In addition to region-specific changes in brain cytokines, we also observe age-specific changes. Within a given brain region, the profile of cytokines that is altered in MIA offspring is distinct at each age. This dynamic response is not unexpected since cytokines are part of both positive and negative feedback loops that regulate each other's expression and function to keep immune responses within a homeostatic range.

Individual cytokines do not work in isolation, but rather in complex networks (Careaga et al., 2010). Interestingly, we find no obvious pattern in the age-specific changes in terms of alternating pro-or anti-inflammatory cytokines. However, classifying cytokines in this way is only partially useful as most cytokines can be either pro-or anti-inflammatory depending on the cellular context and the levels of other cytokines. Moreover, the biological effects of individual cytokine levels can vary widely—a small change in levels of some cytokines can cause dramatic physiological effects, while large changes in others may have minimal outcomes. Taken together, these properties caution against general conclusions about the effects of MIA-induced changes in cytokines on inflammation in the brain.

Another indication that cytokines may not have traditional pro-or anti-inflammatory roles in MIA brains is that there is no overt evidence of inflammation in any brain region at any age examined, even at times when traditionally pro-inflammatory cytokines are elevated. The lack of immune cell infiltration into the brain, combined with little evidence of changes in gross BBB permeability, suggests that poly(I:C) MIA does not cause inflammation in the postnatal brain in the classic sense. Consistent with this interpretation, there is also no change in microglial density in any of the three brain regions examined and there is no qualitative change in microglial morphology in MIA brains (**Fig. 7**). These results are also consistent with a lack of immune cell infiltration, but inconsistent with reports of microglial activation in postmortem brain tissue from ASD individuals (Vargas et al., 2005). Since increased brain cytokines cannot be attributed to infiltrating immune cells, it is possible that any of the cell types endogenous to the brain could be responsible. Prior work has shown that neurons, microglia, and

astrocytes can produce cytokines during normal development *in vivo* (Deverman and Patterson, 2009). Although cytokines could in principle also come from the periphery by crossing the BBB, the postnatal BBB is relatively impermeable to most cytokines (Erickson and Banks, 2011) and MIA-induced changes in brain cytokines are region-specific and do not correlate with serum cytokines at any age (**Fig. 6**). Identifying the source and targets of the MIA-induced cytokines will be important for understanding the consequences of these changes on neuronal connectivity and potentially glial function.

Both epidemiological and experimental results indicate that the effect of MIA on neuropathology and behavior in offspring is determined by the timing of the infection. Maternal viral infection during the late first and early second trimesters, in particular, is associated with increased risk of ASD (Atladottir et al., 2010; Brown 2012; Hagberg et al., 2012; Patterson, 2011a). Similarly, infection during early to mid-pregnancy is related to an increase in risk for SZ in offspring (Brown et al., 2004; Brown 2012). Moreover, differential effects of Poly(I:C) injection during early and late gestation on neuropathology and behaviors in offspring have been found in the MIA mouse model (Meyer et al 2006b). Our experiments used poly(I:C) injection at E12.5, since this stage of gestation correlates with the late first trimester in humans (Clancy et al 2007)—the time that infections are most closely linked to increased incidence of ASD and SZ (Atladottir et al. 2010, Brown et al. 2004).

Remarkably, many of the cytokines altered in the MIA brains and serum are similar to those found to be altered in ASD and SZ (Bauer et al., 2007; Brown and Patterson, 2011; Careaga et al., 2010; Nawa et al., 2000). Significant increases in plasma cytokine levels (IL-1β, IL-6, TNFα, IFNγ, IL-8, IL-12p40) have been reported in children

diagnosed with ASD when compared with children without a family history of ASD (Ashwood et al., 2011; Molloy et al., 2006; Vargas et al., 2005). Most of these cytokines are also increased at P7 in MIA offspring serum (**Figs. 5,6**). Chemokines, including Eotaxin, RANTES, and MCP-1, are also elevated in the serum of autistic children (Ashwood et al., 2011) and all of these are elevated in MIA serum at P7 (**Fig. 6**). Neural inflammation, marked by increased pro-inflammatory cytokines and chemokines, including IFN-γ, IL-1β, IL-6, IL-12p40, TNF-α, and MCP-1, is also found in postmortem brain tissue from individuals with ASD over a wide range of ages (Vargas et al., 2005). Although we found changes in many of these cytokines in the MIA mouse brain, it is difficult to directly compare these results because of the wide range of ages examined in the human study (4–45 years of age). Similar findings in SZ also indicate elevated levels of cytokines in the blood of patients, including elevations in IL-1β, IL-6, IL-12, IFNγ, TNFα, and RANTES (Kunz et al., 2011; Yao et al., 2003), although many of these studies did not control for secondary variables and often report conflicting results. The fact that the poly(I:C) model displays several cytokine changes in common with both SZ and ASD reinforces the construct validity of the model, given that these disorders share the risk factor of maternal infection and that their psychiatric and anatomical pathologies significantly overlap (Lord et al., 2000; Meyer et al., 2011; Rapoport et al., 2009). How the risk of these distinct disorders is increased in humans via a single environmental risk factor is currently unknown but is likely to involve genetic susceptibility and/or the timing and intensity of the infection (Fatemi et al., 2008; Meyer et al., 2006a,b). Identifying how this large family of signaling molecules, the cytokines, are altered over time in brain development by infection and other environmental risk factors may

highlight targets for novel diagnostic tests and new immune-based therapies for ASD and SZ in the future.

Methods

MIA for cytokine measurements

Female C57BL/6J mice (Charles River; Wilmington, MA) were obtained from the Caltech breeding facility and housed on a 12:12 h light:dark cycle at 29 ± 1 °C with food and water available *ad libitum*. Mice were mated overnight, and the presence of a vaginal plug on the following morning was noted as E0.5. Pregnant **mice** were injected intraperitoneally (i.p.) on E12.5 with saline or poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO). E12.5 was chosen since this stage of gestation correlates with the late first trimester in humans (Clancy et al 2007)—the time that infections are most closely linked to increased incidence of SZ and ASD (Atladottir et al. 2010, Brown et al. 2004). Poly(I:C) was freshly dissolved in saline and administered i.p. at 20 mg/kg based on the weight of the poly(I:C) itself, not including the total weight of the potassium salts. Control mice were injected with saline alone at 5 µl per gram body weight. This concentration of Poly(I:C) is higher than that used for intravenous injections (Meyer et al. 2006) and was selected because it is the optimal i.p. dose that causes MIA, while preserving viability of offspring (Ito et al. 2010).

Blood collection and brain dissections

Control and poly(I:C) offspring were sacrificed at 5 ages: P0, P7, P14, P30, and P60. Both male and female offspring were used in this study. Mice were deeply anesthetized

with Nembutal (10 µl/g). At least 150 µl of blood/animal was first collected by cardiac puncture and transcardial perfusion was then performed using 10-30 ml of sterile PBS (by weight). P0 offspring were processed without perfusion. Whole brains were quickly removed and placed in ice-cold Earle's balanced salt solution for microdissection of the FC, CC, and HC. All tissues were snap-frozen in liquid nitrogen and stored at −80°C. Both blood and brains were sent overnight on dry ice to U.C. Davis for processing.

Sample processing

Blood was centrifuged (12,000 x g, 4°C, 20 min) to obtain serum, which was then stored at −80°C. Frozen tissues were thawed and disrupted in Bioplex cell lysis buffer (BioRad) containing factors 1 and 2 (protease and phosphatase inhibitors, respectively; BioRad) and the protease inhibitor phenyl-methylsulfonyl fluoride (500 mM; Sigma–Aldrich). A small plastic pestle was used to homogenize the samples. Tissue was further homogenized by trituration using 200 µl pipette tips. The homogenate was then agitated for 30-40 min on ice and centrifuged at 4°C and 6000 x g (Eppendorf centrifuge 5417R) for 20 min. The supernatant was removed and aliquots stored at −80 °C. The protein content of each sample was determined using the BioRad Protein Assay (BioRad), with bovine serum albumin as a standard, according to the manufacturer's protocol. Sample absorbances were read at 560 nm using a spectrophotometer (Perkin Elmer HTS7000).

Cytokine measurements

BioRad (Hercules, CA) 23-plex mouse kits were used for all assays. Assays were performed according to the manufacturer's instructions. Reagents were kept on ice until

use, minimizing exposure of the beads to light. All samples were run in duplicate and were assayed with the BioRad cytokine reagent kit and either the diluent kit for serum samples or the cell lysis kit for tissue samples. All buffers and diluents were warmed to room temperature prior to use. Lyophilized cytokine standards (containing IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, Eotaxin, G-CSF, GM-CSF, IFNγ, KC, MCP-1, MIP 1α, MIP1β, RANTES, and TNF-α) were reconstituted first to a master standard stock using 500 µl of diluent. Nine concentrations of the standards were made by eight, three-fold serial dilutions of the master standard stock in either cell lysis buffer (for brain tissue) or serum diluent. Samples were run at 200 µg protein/well for the mouse brain homogenates. Serum samples were diluted 1:3 prior to assay. All samples were held at 4°C for 10 min before the start of the assay. Corresponding buffer blanks were run to determine the level of background. All the wash steps were performed on a Bio-Plex Pro Wash Station at room temperature. The plates were then read in the Bio-Plex 200 System and the data analyzed using BioPlex Manager 4.1 software with 5-parameter logistic regression (5PL) curve fitting to determine the standard curve from the 8 standards in duplicate and extrapolate the sample concentrations from the standard curve. The goodness-of-fit for each point on the standard curve was determined by the BioPlex Manager software. The standard recovery, calculated by taking the ratio of the calculated concentration value divided by the expected amount of standard, was within an acceptable range of recovery of between 70-130% for all of the standards used. Only standards and samples with coefficients of variance under 5% were used. Readings were excluded if they were at or below the background value.

MIA for immunohistochemistry

Female C57BL/6J mice (Charles River) were housed at U.C. Davis in accordance with the protocol approved by the Institutional Animal Care and Use Committee. Mice were mated overnight and the presence of a vaginal plug on the following morning was noted as day E0.5. Pregnant mice were injected (in the same fashion as for MIA for cytokine measurements described above) on E12.5 with saline or poly(I:C). Brains of offspring at the five ages were dissected into the three regions and post-fixed for 3 hrs in 4% paraformaldehyde in PBS. The paraformaldehyde was then replaced with 30% sucrose and left overnight at 4^{o} C. The sucrose solution was changed twice until the brains sank. The brains were then frozen in OCT on dry ice and cut into 30 μm sections using a freezing microtome (Leica). The sections were blocked with 5% horse serum + 0.025% Triton in PBS for 2 hrs at room temperature, and then stained with primary antibodies, all at 1:1000: rabbit anti-IBA1 (WAKO, Japan), anti-CD45R (Abcam), rat anti-CD3 (R&D Systems), rat anti-GR1 (R&D Systems) at 4°C for 48 hrs. Sections were washed three times for 10 min each in PBS while shaking. Sections were incubated in secondary antibody for 90 min (1:200; Cy2 anti-rabbit, Cy3 anti-rabbit, and Cy2 anti-rat, Vector Laboratories) and mounted with Vectashield mounting media containing DAPI (Vector Laboratories) on 1% gelatin-coated slides.

Imaging

For assessing immune cell-infiltration, immunostaining was imaged with a 40x 1.2 NA objective on an epifluorescent microscope (Nikon E600, Nikon, Tokyo) under a mercury

arc lamp using computer software to run the camera (a Spot View Advanced 2.0 (Diagnostic Imaging Inc.) For microglia, immunostaining was imaged using a 1.2 NA 40x oil objective on an Olympus Fluoview 2.1 laser-scanning confocal system.

Stereology

The density of Iba-1-positive microglia was quantified using stereological methods (Feng et al., 2011). Every third section (of nine sections) were used for a total of three sample sections per brain region. The Iba-1 cell counts were made on an epifluorescent microscope (Nikon E600, Nikon, Tokyo) and analyzed using Stereologer computer software (version 1.3, Systems Planning & Analysis, Inc., Alexandria, VA). Microglia cell counts were taken from FC layer 2-3, CC layer 2-3, and HC area CA1 of the stratum radiatum at five ages (P0, P7, P14, P30, P60). Microglia were identified by Iba-1-positive cell body staining. The brain region of interest was outlined using a 4x (0.67 NA) objective. Microglia identification and counting were performed with a 100x oil (1.2 NA) objective.

Blood-brain barrier integrity

25 mg/kg Evan's blue dye was injected i.p. in offspring at the five ages examined. Four hours later, the brain was removed, rinsed in PBS, examined using a dissecting microscope (Olympus CK30-F100), and imaged using a digital camera (Olympus FE-370). Changes in the amount of Evans Blue signal were compared qualitatively.

Statistics

Unpaired t-tests were used to determine statistical significance (* = $p < 0.05$) using GraphPad Prism software.

Behavioral Testing

Behavioral tests were conducted on mice starting at six weeks of age according to previously published methods (Smith et al., 2007). Mice were tested for PPI of acoustic startle using SR-LAB startle chambers (San Diego Instruments). Mice were acclimated to the chambers for 5 minutes before exposure to 6, 120 db tones (startle stimulus). They were then subjected to 14 randomized blocks of either no startle, startle only, startle preceded by a 5 db pre-pulse, or startle preceded by a 15 db pre-pulse. PPI is defined as the percentage of (startle only – 5 or 15 db pre-pulse)/startle only. For LI testing, mice were placed in chambers with parallel-grid shock floors (Coulbourn Instruments). On the first day of testing, mice were presented with a pre-exposure to 40, 30 sec tones followed by three pairings of the tone with a mild foot shock. Non-pre-exposed mice are presented with the three pairings only. On the second day, mice were placed in the same chambers for 8 min to record freezing in response to the context. On the third day, mice were placed back in the same chambers and presented with an 8 min tone. LI is defined as the difference in freezing in response to the tone in pre-exposed mice compared to non-pre-exposed mice.

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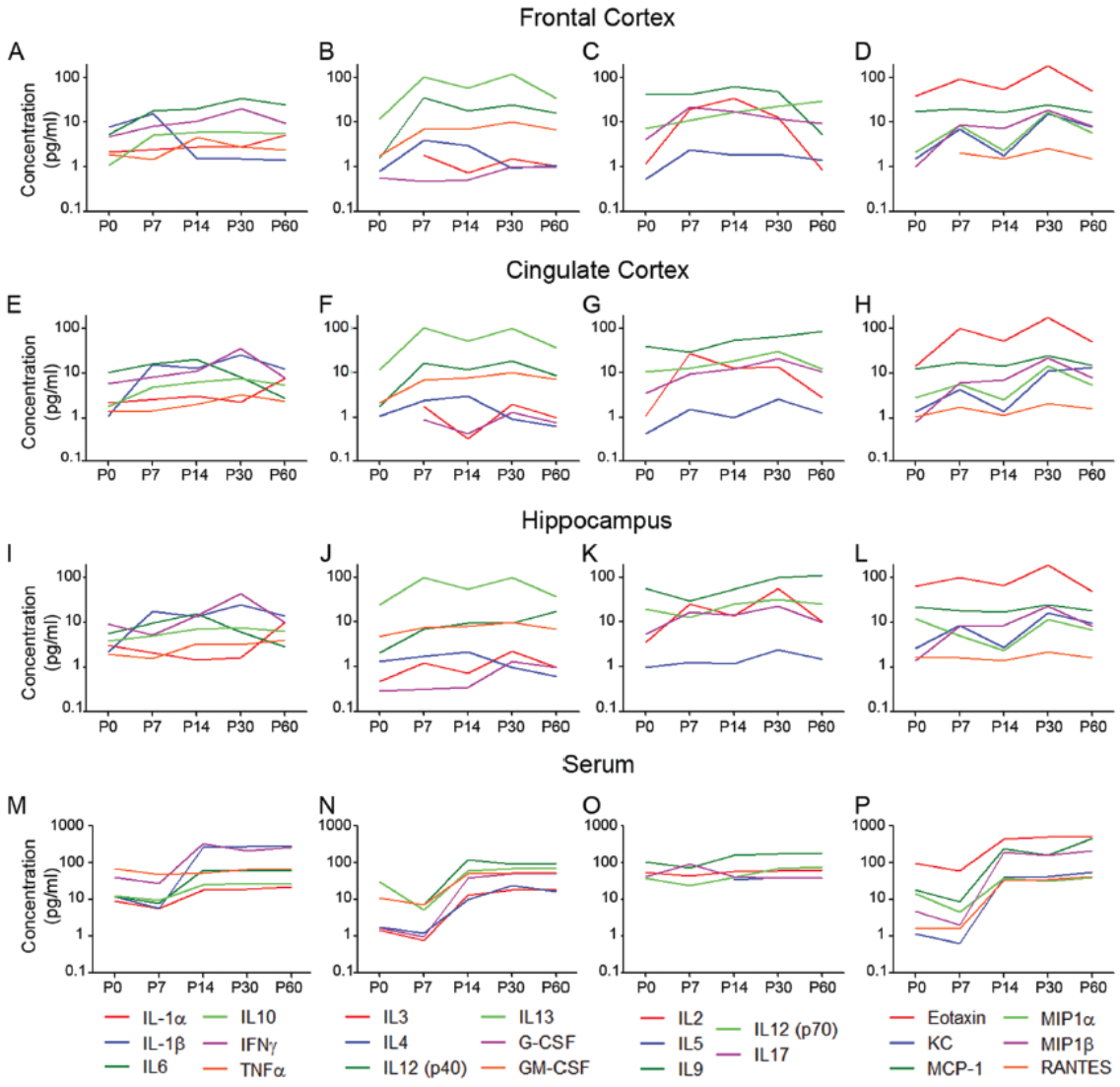


Figure 1. Many cytokines are expressed in serum and brain throughout normal development. Cytokine concentrations in the typically-developing brain change with age and some are region-specific. To illustrate changes in cytokine concentrations with age, the average values for each cytokine concentration are plotted at the five ages examined. Cytokine concentrations are plotted in pg/mg brain tissue (A-L) and pg/ml serum (M-P) for FC (A-D), CC (E-H), HC (I-L), and serum (M-P). The 23 cytokines are separated into 4 groups: (1) commonly-studied brain cytokines (A,E,I,M) including IL-1α, IL-1β, IL-6, IL-10, IFNγ, and TNFα, (2) a first set of additional cytokines with similar step-like

changes in serum concentration with age (B,F,J,N) including IL-3, IL-4, IL-12(p40), IL-13, G-CSF, and GM-CSF, (3) a second set of additional cytokines with stable levels in serum (C,G,K,O) including IL-2, IL-5, IL-9, IL-12(p70), and IL-17, and (4) chemokines (D,H,L,P) including Eotaxin, KC, MCP-1, MIP1α, MIP1β, and RANTES. Error bars are not included in these graphs to enhance visibility of the trends in expression, however all values of mean ± SEM concentration are included in Suppl. **Tables 1 and 2.**

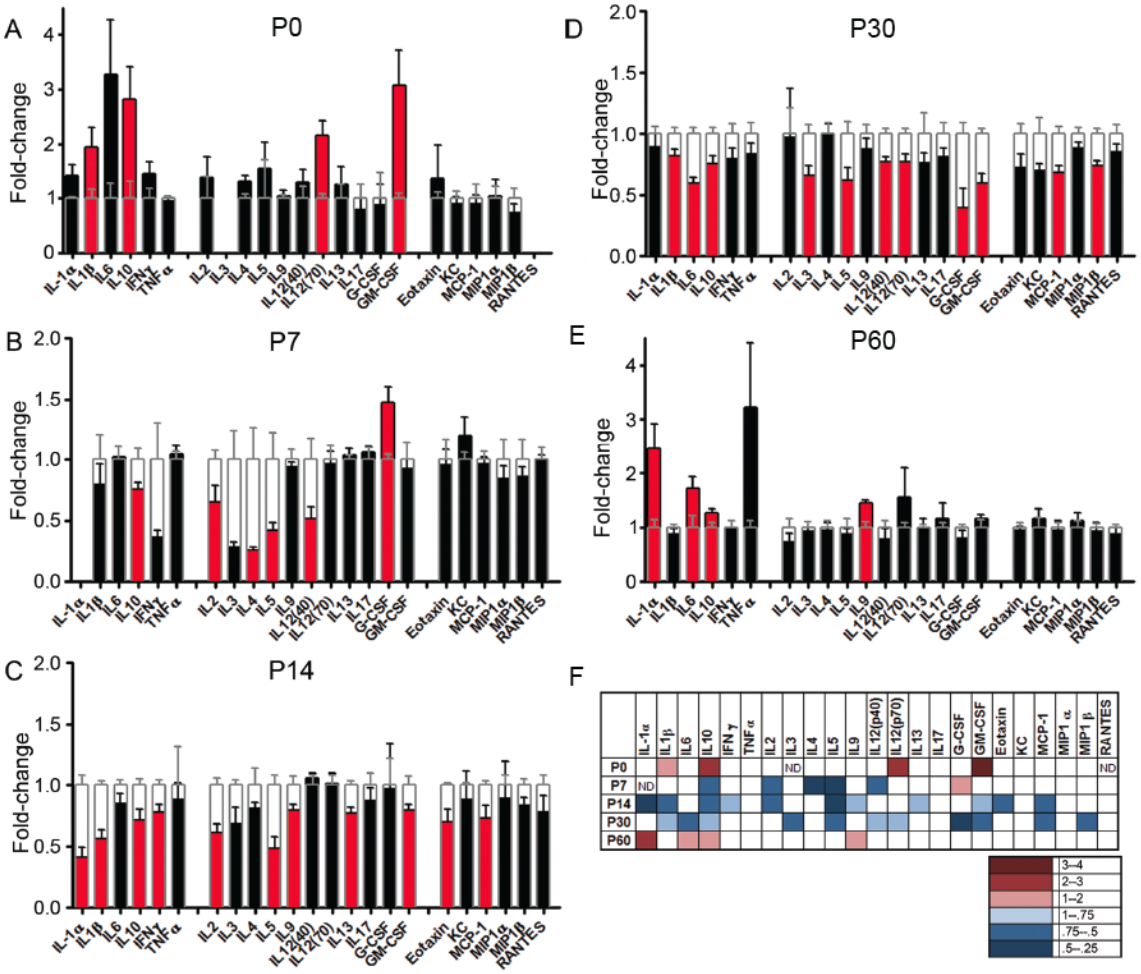


Figure 2. MIA induces long-lasting changes in cytokines in frontal cortex throughout development. The fold-change ± SEM in cytokine concentration in MIA brain regions compared to control values is plotted. Values from MIA brains are either black or red, and the control values are superimposed in clear bars outlined in grey. Red bars indicate statistically significant changes compared to controls ($p < 0.05$). Increases are shown above 1 and decreases, below 1. (A) At P0, MIA-induced increases are found for four cytokines. IL-3 and RANTES are below the level of detection at this age. (B, C, D) At P7, P14 and P30, many cytokines in MIA FC are lower compared to controls. (E) At P60, fewer cytokines in MIA FC are altered and all of these are elevated. (F) A heat

map is used to summarize the results for MIA-induced changes in cytokines at all ages examined. Statistically significant changes are indicated in color: red indicates increases, and blue decreases, in cytokine levels compared to controls. The degree of change is indicated by the depth of color as indicated in the lower panel. ND = below the level of detection. n = 5–6 brains per treatment group (pooled from at least two independent litters).

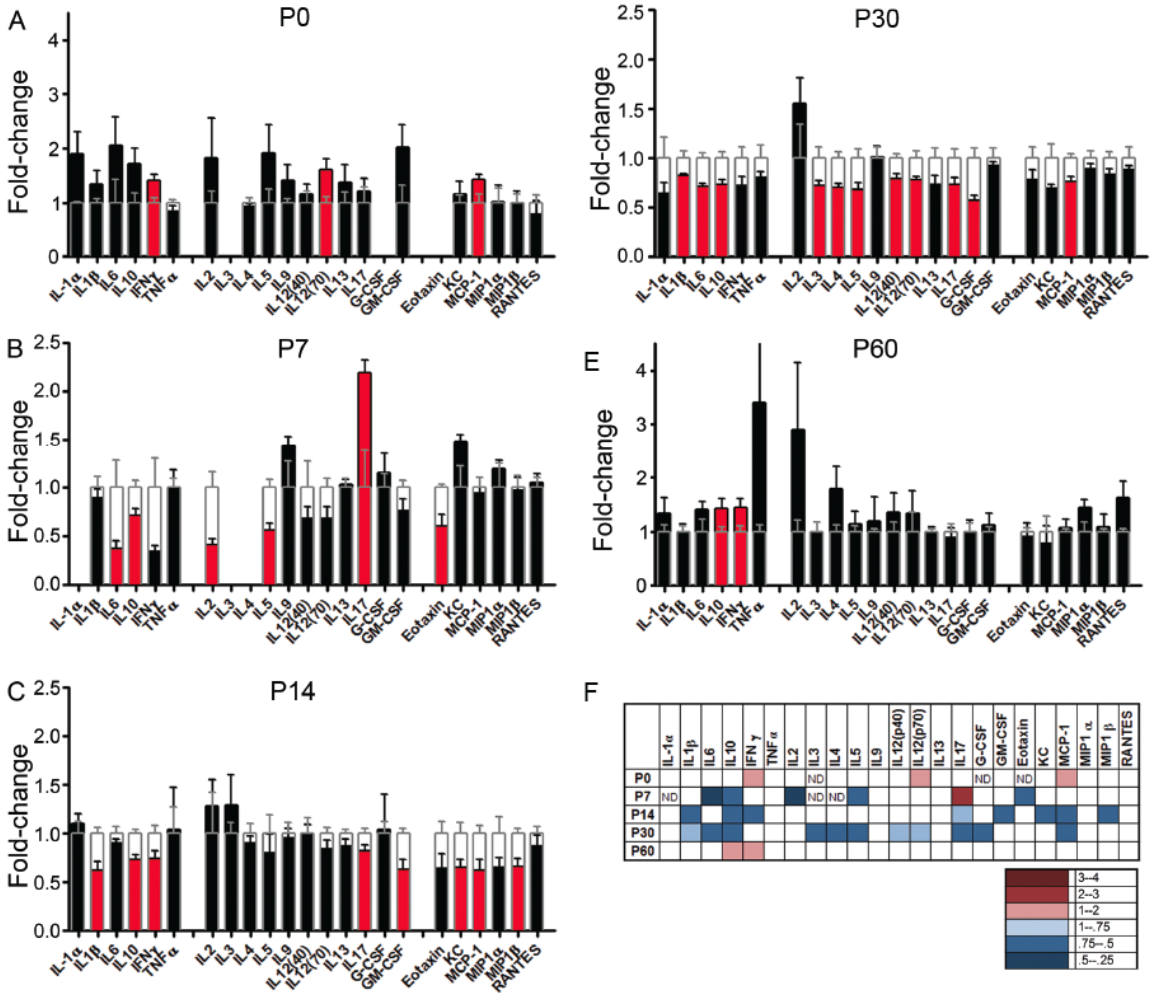


Figure 3. MIA induces long-lasting changes in cytokines in cingulate cortex throughout development. Data is plotted as in Fig. 2. (A) At P0, MIA induces increases in three cytokines. (B) At P7, five cytokines are lower than controls, while IL-17 is the only elevated cytokine at this age. IL-1α, IL-3, and IL-4 are below the level of detection. (C) At P14, eight cytokines are also lower. (D) At P30, eleven cytokines remain lower than controls. (E) At P60, fewer cytokines are altered and both are higher than controls. (F) A heat map is used to summarize the results for MIA-induced changes in cytokines at all ages examined. Statistically significant changes are indicated in color: red indicates increases and blue decreases in cytokine levels compared to controls. The degree of

change is indicated by the depth of color as indicated in the lower panel. ND = below the level of detection. n = 5–6 brains per treatment group (pooled from at least two independent litters).

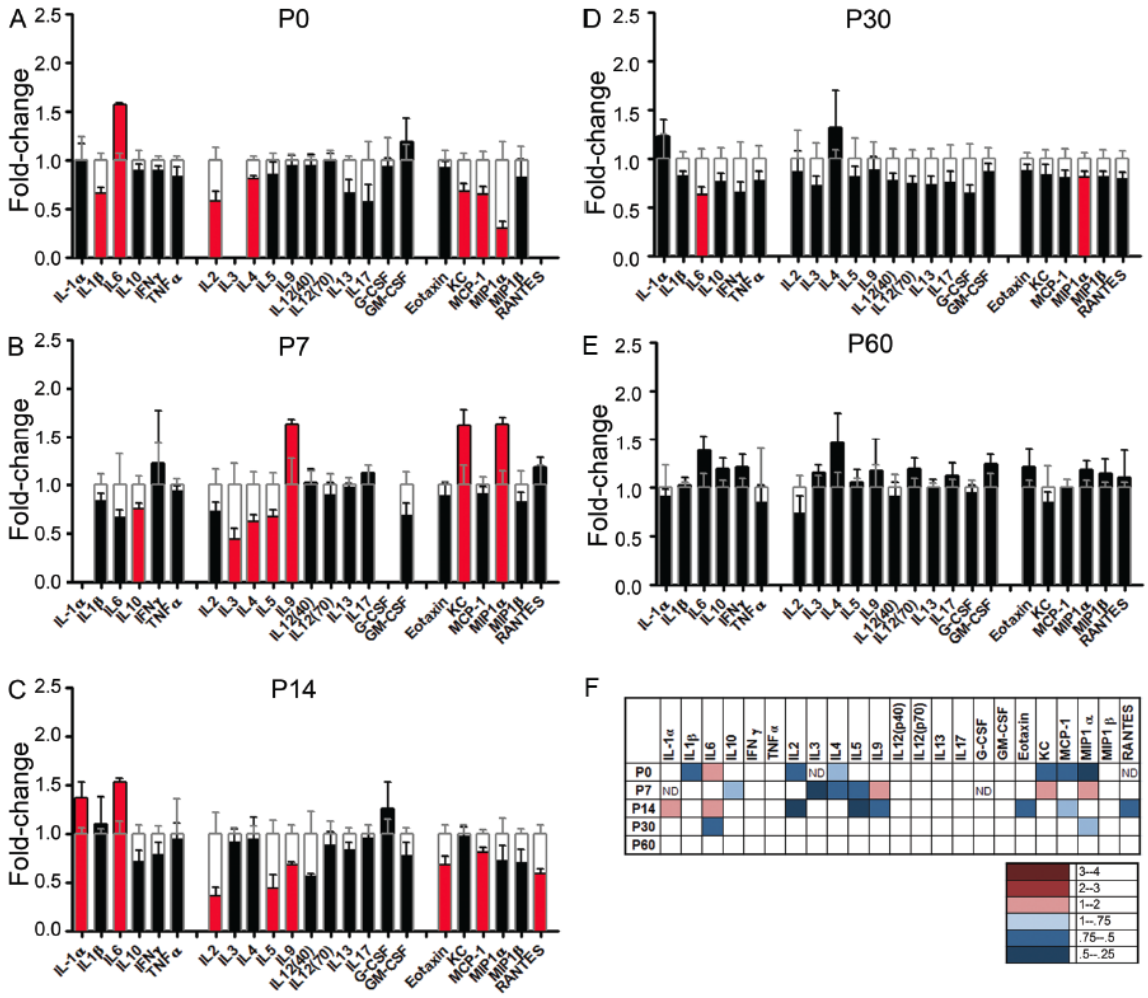


Figure 4. MIA induces long-lasting changes in cytokines in hippocampus throughout development. Data is plotted as in Fig. 2. (A) At P0, MIA induces higher levels of IL-6 and lower levels of six other cytokines, while MIP1α, IL-3, and RANTES are below the level of detection. (B) At P7, three cytokines are elevated, while four are lower than controls. IL-1α and G-CSF are below the level of detection. (C) At P14, IL-1α and IL-6 are higher and six other cytokines are lower than controls. (D) At P30, IL-6 and MIP1α are lower than controls. (E) At P60, no cytokines levels are changed relative to controls. (F) A heat map summarizes the results for MIA-induced changes in cytokines at all ages

examined. Statistically significant changes are indicated in color: red indicates increases and blue, decreases in cytokine levels compared to controls. The degree of change is indicated by the depth of color as indicated in the lower panel. ND = below the level of detection. n = 5–6 brains per treatment group (pooled from at least two independent litters).

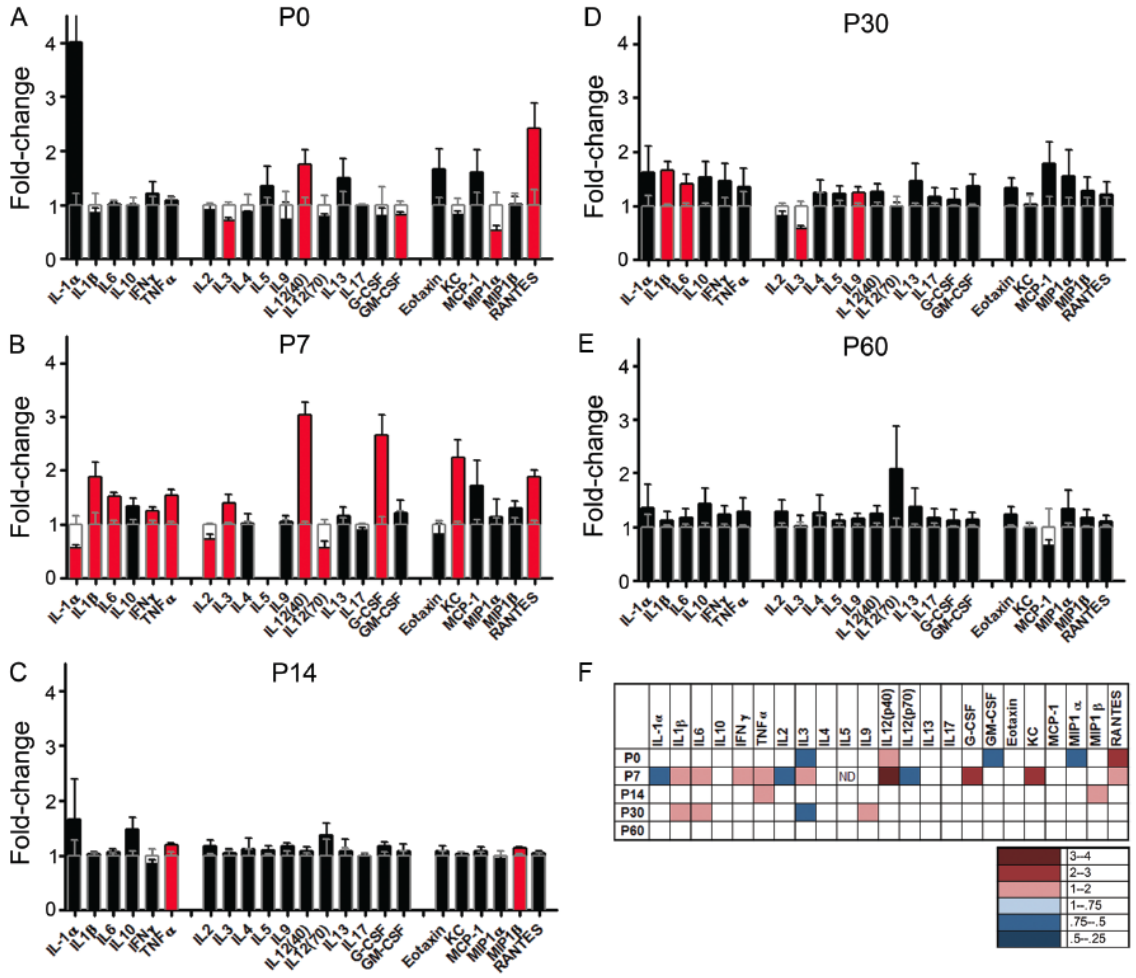


Figure 5. MIA induces long-lasting changes in cytokines in serum throughout development. Data is plotted as in Fig. 2. (A) At P0, MIA increases two cytokines and decreases three. (B) At P7, MIA increases levels of nine cytokines and decreases three. IL-5 is below the level of detection at this age. (C) At P14, two cytokines are higher than controls. (D) At P30, three cytokines are increased and one is decreased. (E) At P60, no cytokines levels are changed relative to controls. (F) A heat map to summarize the results for MIA-induced changes in cytokines at all ages examined. Statistically significant changes are indicated in color: red indicates increases, and blue decreases, in cytokine

levels compared to controls. The degree of change is indicated by the depth of color as indicated in the lower panel. ND = below the level of detection. n = 5–6 brains per treatment group (pooled from at least two independent litters).

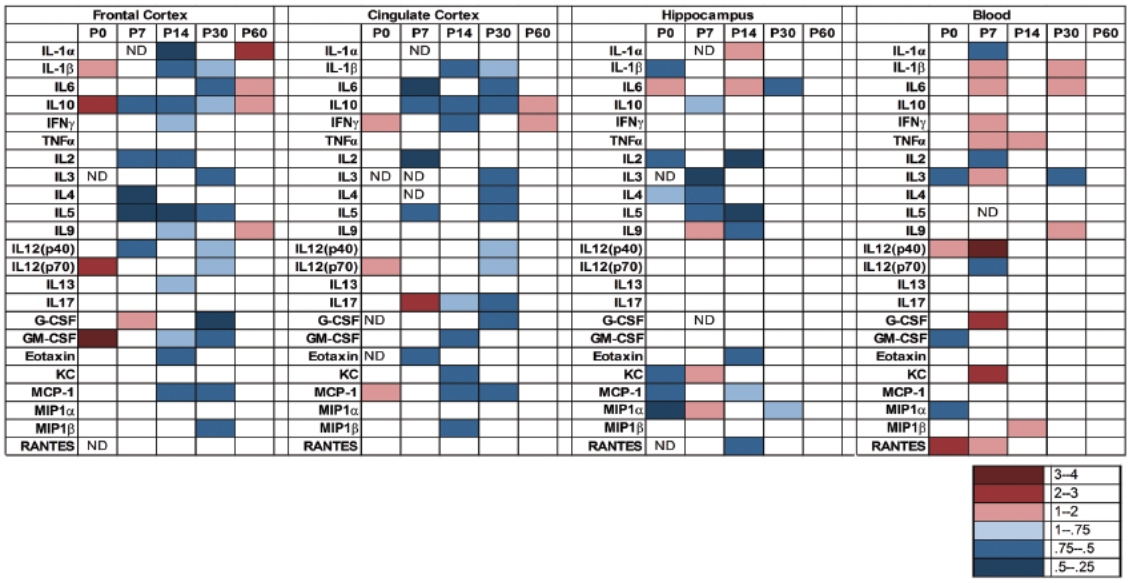


Figure 6. Summary of cytokine changes in brain and serum across development.

A heat map is used to summarize the results for MIA-induced changes in cytokines at all ages examined. Statistically significant changes are indicated in color: red indicates increases and blue indicates decreases in cytokine levels compared to controls. The degree of change is indicated by the depth of color as indicated in the lower panel. This figure clearly shows the similar overall pattern in MIA-induced cytokine changes in FC and CC during development, characterized by increases in cytokines at birth (P0) and in the adult (P60), and dramatic decreases in many cytokines during periods of synaptogenesis and activity-dependent plasticity (P7-P30). This pattern is distinct from that in HC, where there are both increases and decreases in cytokine in MIA offspring at all ages examined. Finally, MIA-induced changes in serum cytokines do not correlate with changes in brain cytokines. ND = below the level of detection.

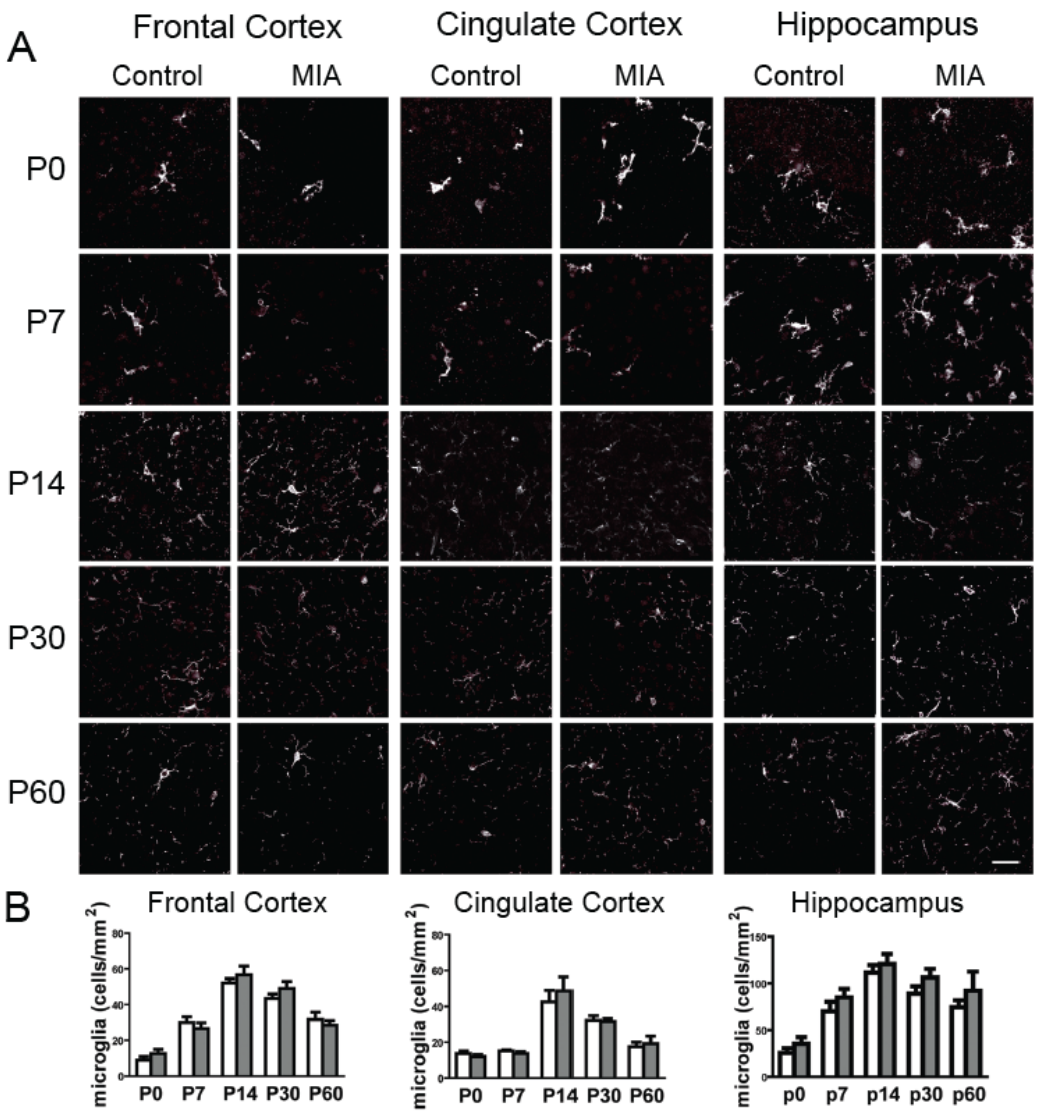
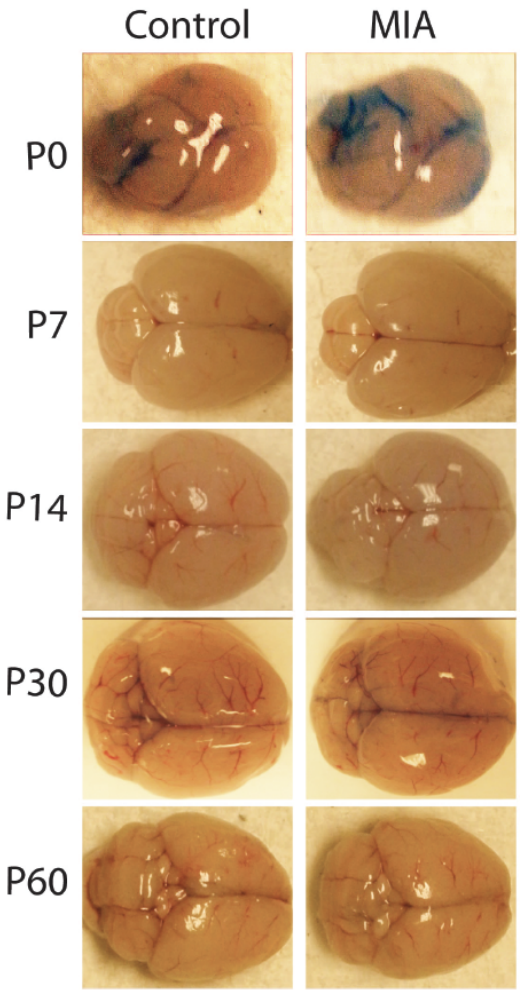


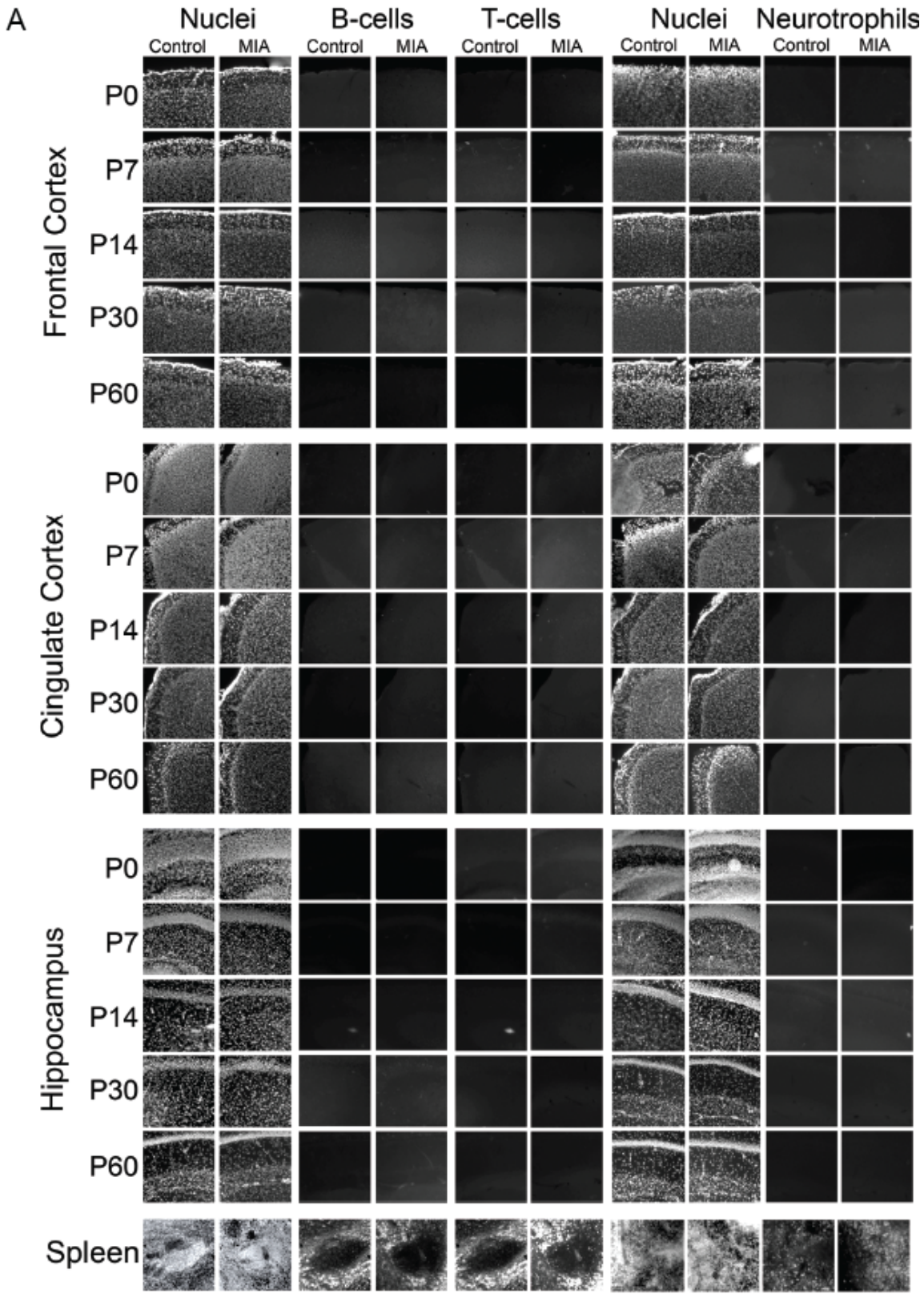
Figure 7. MIA does not alter microglial number or morphology.

The density of Iba1 + microglia (white) was quantified using Stereologer in each brain region at each age examined. (A) Representative images of coronal sections taken at 40X through FC, CC, and HC at five ages during postnatal development. (B) Quantification of the density of Iba1+ microglia for each brain region over age. The density generally increases from birth to P14 and then decreases to intermediate levels in the adult. There is no difference in microglial density between MIA and control brains. Open bars indicate control values and gray bars, MIA values.



Supplemental Fig. 1. MIA does not cause major breaches in the BBB.

Representative brains from control and MIA offspring are shown for each of the four ages examined, following Evans Blue staining to detect BBB permeability. There is no qualitative difference in BBB permeability in MIA brains compared to controls at any age examined.


A
Nuclei
B-cells
T-cells
Nuclei
Neurotrophils
Control
MIA
Control
MIA
Control
MIA
Control
MIA
Control
MIA
Frontal Cortex
P0
P7
P14
P30
P60
Cingulate Cortex
P0
P7
P14
P30
P60
Hippocampus
P0
P7
P14
P30
P60
Spleen
B Spinal Cord following EAE
Nuclei
B-cells
T-cells
Nuclei
Neutrophils

Supplemental Fig. 2. MIA does not induce immune cell infiltration in the CNS of offspring.

(A) Representative images of coronal sections taken at 10X through the three brain regions are shown at five different ages. Sections were stained with antibodies specific for B cells, T cells, and neutrophils. No immunoreactivity is seen over that detected in the no-primary antibody control. Adult spleen is included as a positive control and, as expected, all three cell types are visible in spleen. (B) Spinal cord sections from mice subjected to experimental autoimmune encephalitis (EAE) are included as a second positive control for our ability to detect immune cell infiltration. All immune cell types are stained, at the expected low levels, in EAE spinal cord.

Supplemental Table 1 (Dataset online). Cytokine concentrations in FC and CC. Average concentrations (pg/ml for brain regions and pg/ml for serum) ± SEM for 23 cytokines are shown for saline (control) and poly(I:C) (MIA) conditions for each brain region at each age. The fold-change in cytokines resulting from MIA was calculated as the ratio of the average values for poly(I:C)/saline (P/S). P-values for t-tests between MIA and controls for each comparison are also indicated. Grey fills indicate cytokines that are significantly different between MIA and control values ($p<0.05$). ND = not detected.

Supplemental Table 2 (Dataset online). Cytokine concentrations in HC and Serum. Average concentrations (pg/ml for brain regions and pg/ml for serum) ± SEM for 23 cytokines are shown for saline (control) and poly(I:C) (MIA) conditions for HC and serum at each age. The fold-change in cytokines resulting from MIA was calculated as the ratio of the average values for poly(I:C)/saline (P/S). P-values for t-tests between MIA and controls for each comparison are also indicated. Grey fills indicate cytokines that are significantly different between MIA and control values ($p<0.05$). ND = not detected.

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Chapter 9

Maternal immune activation alters nonspatial information processing in the hippocampus of the adult offspring

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Abstract

The observation that maternal infection increases the risk for schizophrenia in the offspring suggests that the maternal immune system plays a key role in the etiology of schizophrenia. In a mouse model, maternal immune activation (MIA) by injection of poly(I:C) yields adult offspring that display abnormalities in a variety of behaviors relevant to schizophrenia. As abnormalities in the hippocampus are a consistent observation in schizophrenia patients, we examined synaptic properties in hippocampal slices prepared from the offspring of poly(I:C)and saline-treated mothers. Compared to controls, CA1 pyramidal neurons from adult offspring of MIA mothers display reduced frequency and increased amplitude of miniature excitatory postsynaptic currents. In addition, the specific component of the temporoammonic pathway that mediates object-related information displays increased sensitivity to dopamine. To assess hippocampal network function in vivo, we used expression of the immediate-early gene, c-Fos, as a surrogate measure of neuronal activity. Compared to controls, the offspring of poly(I:C)-treated mothers display a distinct c-Fos expression pattern in area CA1 following novel object, but not novel location, exposure. Thus, the offspring of MIA mothers may have an abnormality in modality-specific information processing. Indeed, the MIA offspring display enhanced discrimination in a novel object recognition, but not in an object location, task. Thus, analysis of object and spatial information processing at both synaptic and behavioral levels reveals a largely selective abnormality in object information processing in this mouse model. Our results suggest that altered processing of object-related information may be part of the pathogenesis of schizophrenia-like cognitive behaviors.

Introduction

Schizophrenia is a major psychiatric disorder with a strong genetic contribution (Burmeister et al., 2008; Bertolino and Blasi, 2009). Nonetheless, epidemiologic evidence indicates that genetic factors alone cannot explain the pathogenesis. For example, the concordance for schizophrenia in monozygotic twins is approximately 48% (Gottesman, 1991). Furthermore, while the concordance between monozygotic twins who share a placenta is 60%, the concordance rate between such twins who do not share a placenta is only 10% (Davis et al., 1995; Patterson, 2007). These studies suggest the importance of the fetal environment. Supporting this idea, Mednick et al. (1988) reported that fetuses gestating during a viral epidemic are at elevated risk for developing schizophrenia. Subsequent prospective studies have shown that maternalinfections of various types increase the risk for schizophrenia in the offspring 3to 7-fold (reviewed by Patterson, 2009; Brown and Derkits, 2010).

Based on this evidence, several animal models of MIA have been established (reviewed by Meyer et al., 2009; Patterson, 2009). Among these, administration of the synthetic dsRNA, poly(I:C) can effectively induce MIA, resulting in offspring that display a variety of behaviors and neuropathologies that are consistent with those seen in schizophrenia patients (Meyer and Feldon, 2009; Patterson, 2009). Thus, this animal model is useful for investigating the pathophysiology of schizophrenia.

Clinical studies reveal an important role for dopamine (DA)mediated signaling in the pathophysiology of schizophrenia. For example, drugs that increase DA release induce several aspects of schizophrenic psychosis in normal adults, and exacerbate psychotic symptoms in patients with schizophrenia (Angrist and Vankammen, 1984;

Lieberman et al., 1987). Moreover, all drugs currently in widespread use for the treatment of schizophrenia block DA D2 receptors (Creese et al., 1976). Other studies suggest that there is a deficit in DA D1 receptor-mediated transmission in prefrontal areas of schizophrenic patients (Davis et al., 1991; Toda and Abi-Dargham, 2007). Indeed, imaging studies of patients reveal an increased D2 receptor density in the striatum (Weinberger and Laruelle, 2001) and a decreased D1 receptor density in the prefrontal cortex (Okubo et al., 1997).

Deficits in other cortical regions may also play a key role in the pathophysiology of schizophrenia. Among them, hippocampal abnormalities are commonly found (Heckers and Konradi, 2002). Lipska et al. (1993) suggested that the important variable is the developmental period during which the hippocampal damage takes place, because lesion of the adult rat hippocampus fails to produce schizophrenia-like behaviors, while hippocampal disruption in neonatal stages causes these behavioral alterations to emerge in adulthood. Most importantly in the present context, DA D1and D2-like receptors are highly expressed in the hippocampus (Wamsley et al., 1989; Meador-Woodruff et al., 1994; Khan et al., 1998) and a reciprocal functional interaction between the DA system and the hippocampus has been suggested (Lisman and Grace, 2005). Together, these studies indicate that hippocampal dysfunction participates in the pathogenesis of schizophrenia.

Considering these findings, we used the poly(I:C) MIA mouse model to investigate the pathogenesis of schizophrenia-like behaviors. We focus on the hippocampal network and the influence of DA, conducting experiments at the synaptic as well as the behavioral level. The data suggest a link between synaptic dysfunction, DA

and altered behavior.

Results

CA1 pyramidal neurons in the offspring of poly(I:C)-treated mothers display a reduced frequency and increased amplitude of mEPSCs

A number of studies indicate that the brains of schizophrenia patients exhibit a reduction in the volume of the hippocampus (Nelson et al., 1998; Heckers and Konradi, 2002). Although most studies on schizophrenia patients report no significant change in neuronal density in the hippocampus (Dwork, 1997; Harrison, 1999), many post-mortem studies have reported an abnormal expression of synaptic proteins, including synaptophysin (Eastwood and Harrison, 1995; Davidsson et al., 1999), synapsin I (Browning et al., 1993), SNAP-25 (Young et al., 1998) and spinophilin (Law et al., 2004).

Therefore, we asked whether the MIA offspring display abnormalities in synaptic number or efficacy in CA1 pyramidal neurons. We observe an increased amplitude, but decreased frequency in miniature excitatory postsynaptic currents (mEPSCs) (amplitude: saline 8.5 ± 0.3 pA, poly(I:C) 9.9 ± 0.5 pA; frequency: saline 0.95 ± 0.15 Hz, poly(I:C) 0.60 ± 0.05 Hz) (Fig. 1A). There is no significant difference in the kinetics of mEPSC waveforms (Fig. 1B) or membrane properties (membrane capacitance: saline 220 ± 36 pF, poly(I:C) 210 ± 33 pF, membrane resistance: saline 194 ± 62 MX, poly(I:C) 149 ± 55 MX, in mean ± SD), suggesting that the observed differences in mEPSC amplitude and frequency are primarily due to altered synaptic properties. The decrease in mEPSC frequency suggests either presynaptic dysfunction or a reduction in excitatory synapse

number per neuron. To assess presynaptic function, we analyzed paired-pulse facilitation but do not find a significant difference between the groups (saline: 2.1 ± 0.2, poly(I:C): 2.0 ± 0.2; ratio of 2nd to 1st EPSC amplitude) (Fig. 1C), suggesting that presynaptic function in the experimental group is normal and that the reduced mEPSC frequency is likely due to a reduction in excitatory synapse number. The increase in mEPSC amplitude in the offspring of poly(I:C)-treated mothers may be a compensatory response for the reduction of mEPSC frequency (Turrigiano et al., 1998; Sutton et al., 2006). We also tested synaptic plasticity at Schaffer-collateral-CA1 synapses. When LTP was induced by a single train of 100 stimuli at 100 Hz, the magnitude of LTP in slices prepared from the offspring of poly(I:C)-treated mothers is similar to controls (saline: 1.45 ± 0.05, poly(I:C): 1.36 ± 0.07; mean fEPSP slope at 55– 60 min after LTP induction relative to the baseline) (Fig. 1D). Taken together, these results indicate that the adult offspring of poly(I:C)treated mothers display a reduced number of normally functioning excitatory synapses on CA1 pyramidal neurons.

We next performed immunohistochemistry of the synaptic proteins synaptophysin and GluR1 in area CA1. Dendritic distribution of these proteins is similar between control and MIA offspring. In contrast, we found that a cytoskeletal protein, MAP2, shows a distinct distribution along the dendritic axis of CA1 pyramidal neurons in MIA offspring (Supplemental Fig. 1), which is consistent with a study on schizophrenia patients showing an elevated expression of MAP2 in the hippocampus (Cotter et al., 2000).

To examine inhibitory synaptic transmission, we recorded mIPSCs from CA1 pyramidal neurons. We do not find a significant difference in either amplitude or

frequency of mIPSCs between groups (amplitude: saline 13.9 ± 1.1 pA, poly(I:C) 13.6 ± 1.0 pA, frequency: saline 4.9 ± 0.3 Hz, poly(I:C) 4.3 ± 0.5 Hz) (Fig. 1E), suggesting that the function of inhibitory synapses is normal in area CA1.

Temporoammonic-CA1 synapses in MIA offspring display increased sensitivity to dopamine

Many lines of evidence indicate that DA signaling plays an important role in hippocampal function, as well as in the pathophysiology of schizophrenia. For example, DA neurons in the substantia nigra compacta and ventral tegmental area innervate the hippocampus (Swanson, 1987; Gasbarri et al., 1997) and release DA when animals are exposed to novel environments (Ihalainen et al., 1999). This neurotransmitter influences hippocampal synaptic plasticity (Huang and Kandel, 1995; Otmakhova and Lisman, 1996; Li et al., 2003) and frequency-dependent synaptic transmission (Ito and Schuman, 2007). Furthermore, disruption of DA-mediated signaling impairs hippocampal-dependent learning (Gasbarri et al., 1996; El-Ghundi et al., 1999; Rossato et al., 2009). Therefore, we investigated DA-mediated synaptic control in hippocampal area CA1. As previous studies showed a selective influence of DA on the temporoammonic (TA)-CA1 synapses compared to Schaffer-collateral-CA1 synapses (Otmakhova and Lisman, 1999; Ito and Schuman, 2007; Supplemental Fig. 2), we examined DA modulation of TA-CA1 synapses. The TA pathway includes axonal populations from both the medial (MEC) and lateral (LEC) entorhinal cortexes (Steward, 1976; Witter and Amaral, 2004). These projections are topographically organized along the transverse-axis of area CA1, such that the projections from MEC make synapses at proximal CA1 (close to CA3), while

those from the LEC project to distal CA1 (close to subiculum) (Fig. 2A; Steward, 1976; Witter and Amaral, 2004). Previous anatomical studies in the mouse brain showed a topographic projection of the TA pathway similar to that observed in the rat brain (van Groen et al., 2003). Our immunohistochemical analysis of the presynaptic proteins synapsin I and bassoon in the stratum lacunosum-moleculare indeed indicates expression differences between distal and proximal regions of area CA1 (Supplemental Fig. 3). Thus, the presynaptic properties of the proximal and distal TA-CA1 synapses in the topographic projection of entorhinal-cortical fibers may be distinct. This organization of presynaptic proteins is observed in both MIA and control offspring (Supplemental Fig. 3).

We recorded fEPSPs simultaneously from proximal and distal TA-CA1 synapses. The application of DA (20 lM; Otmakhova and Lisman, 1999) to slices prepared from control mice induces a significantly larger depression at proximal TA-CA1 synapses compared to distal TA-CA1 synapses (proximal TA: 57.6 ± 2.2%; distal TA: 65.3 ± 2.3%, relative to baseline; Fig. 2B and 4C). Furthermore, we found that another neuromodulator, norepinephrine (NE), also differentially controls TA synapses made by MEC and LEC inputs (Supplemental Fig. 4). These data indicate that the neuromodulators, DA and NE, can differentially control MEC and LEC inputs to area CA1 of the mouse hippocampus.

In slices prepared from MIA offspring, DA induces a depression comparable to that observed in control slices at proximal TA-CA1 synapses (proximal TA: 55.4 ± 3.1%, relative to baseline; Fig. 2C). At distal TA-CA1 synapses, however, the slices prepared from MIA offspring show a significantly larger depression compared to controls (distal

TA: 56.3 ± 1.4%, relative to baseline; Fig. 2C). To further examine DA sensitivity at distal TA-CA1 synapses, the neurotransmitter was applied sequentially from low to high concentration to acute slices (Fig. 2D) and DA-mediated depression was quantified. We find that, compared to controls, the amount of depression is significantly larger at each DA concentration in the slices prepared from the MIA group (Fig. 2E). These results indicate that the adult offspring of poly(I:C)-treated mothers display an enhanced sensitivity to DA selectively at LEC inputs.

The offspring of poly (I:C)-treated mothers display a distinct c-Fos expression pattern in hippocampal area CA1 following novel object exposure

While the major afferent inputs to the hippocampus are provided by the entorhinal cortex (Cajal, 1911), recent studies demonstrated that two subdivisions of the entorhinal cortex, MEC and LEC, provide distinct information modalities to the hippocampus. Spatial information is carried by axons from the MEC, whereas nonspatial, or object information is carried by axons from the LEC (Hargreaves et al., 2005; Knierim et al., 2006; Manns and Eichenbaum, 2006). Because MIA offspring display higher sensitivity to DA in the LEC projection to area CA1, these animals may exhibit abnormal object information processing.

One of the major features shared by hippocampal and DAreleasing neurons in vivo is the modulation of neuronal activity by stimulus novelty (Knight, 1996; Schultz, 1998; Horvitz, 2000; Rutishauser et al., 2006). Therefore, we examined how hippocampal neurons are activated in vivo during novel object exposure using immunostaining for an immediate-early gene product, cFos (Morgan and Curran, 1991). Immediate-early gene

expression in resting animals is very low (e.g. Supplemental Fig. 5C), but rapidly increases following patterned neuronal activity that induces synaptic plasticity (Cole et al., 1989), suggesting that c-Fos expression can be used as a surrogate marker for synaptic modification (Guzowski et al., 2005). Following accommodation to the home cage for several days, control and experimental mice were exposed to novel objects in the home cage (Fig. 3A). After 2 h of exposure, animals were sacrificed and immunohistochemistry was performed. Control mice show differential c-Fos expression between proximal and distal CA1 pyramidal neurons (Figs. 3B and C and Supplemental Fig. 5A). In contrast, MIA offspring do not show clear differential c-Fos activation between proximal and distal CA1 pyramidal neurons (Figs. 3B and C and Supplemental Fig. 5A). These results suggest that MIA offspring display abnormal object information processing in the hippocampus.

We also examined c-Fos expression after animals were exposed to a novel cage environment. Following accommodation to the home cage for several days, animals were placed in a new cage, which lacked a food box and contained new bedding with a different texture and scent than the prior bedding. After 2 h of such novel location exposure, animals were sacrificed and immunohistochemistry was performed (Fig. 4A). In contrast to the results after novel object exposure (Fig. 3), we observe a similar c-Fos expression pattern in the transverse-axis of area CA1 between the offspring of salineand poly(I:C)-treated mothers (Figs. 4B and C and Supplemental Fig. 5B). Thus, MIA offspring appear to have a selective abnormality in object, but not spatial, information processing, which could be due to hyper-DA sensitivity in LEC inputs at TA-CA1 synapses.

The offspring of poly(I:C)-treated mothers display behavioral inflexibility and abnormal novel object recognition

Our slice recording and c-Fos expression analyses indicate that MIA offspring have a selective abnormality in nonspatial information processing in the hippocampus. To examine if these animals display a corresponding behavioral abnormality, we tested the performance of hippocampus-dependent behavior using the Morris water maze task (Morris, 1984).

We do not find a significant difference in the learning of the initial platform location between experimental and control groups (Fig. 5A and B), suggesting that the MIA offspring have normal ability to acquire spatial navigation memory. After animals learned the initial platform location, we moved the platform to a different location. Two-way ANOVA with session number and prenatal treatment as variables reveals a significant effect of prenatal treatment ($T_{1,222} = 5.693$, $p < 0.05$), as well as a significant effect of session number ($T_{3,222} = 5.875$, $p < 0.01$) with no interaction between the two variables, indicating that while both groups improve their performance over time, the MIA offspring display a significantly slower learning of the new platform location (Fig. 5C). Thus, although the MIA offspring have normal ability to
learn a spatial context per se, they have difficulty in adapting to a change introduced in a previously-learned context.

To further test this idea, we examined how these animals perform in novel object recognition and object location tasks. We placed two different objects in a test cage and allowed animals become familiar with them. One object was subsequently moved to a

new location (object location test) (Fig. 5D). Both groups of animals display a higher preference for the moved object, and we do not find a significant difference in the magnitude of preference between groups (Fig. 5E). In another set of experiments, after familiarization with the two objects, a novel object replaced one of the familiar ones at the same location (novel object recognition test) (Fig. 5D). Although both groups of animals display preference for the new object, the MIA offspring display a significantly stronger preference than control animals (Fig. 5F). Thus, the MIA offspring display abnormally high sensitivity to a novel object, but not to a novel location, which may be due to altered processing of nonspatial information in the hippocampus of these animals. This is consistent with the slice recording and c-Fos findings.

Discussion

Reduced excitatory input on CA1 pyramidal neurons

Our electrophysiological studies demonstrate that the offspring of poly(I:C)-treated mothers display a decreased number, but enhanced efficacy, of excitatory synapses on CA1 pyramidal neurons. Synaptic abnormalities have also been observed in area CA3 in schizophrenia patients, including abnormal mRNA expression of presynaptic proteins (Harrison and Eastwood, 2001).

We do not observe a significant abnormality in the mIPSC amplitude or frequency in the offspring of poly(I:C)-treated animals. Although several studies have reported a decreased number of GABAergic neurons in the hippocampus, most of these differences were observed in area CA2/3, not in area CA1 (Benes et al., 1996, 1997; Heckers and

Konradi, 2002). Thus, GABAergic input to area CA1 pyramidal neurons appears normal in both our model and in patients.

Increased dopamine sensitivity in the temporoammonic pathway

Compared to the Schaffer-collateral pathway in area CA1, the TA pathway has been a relatively unexplored circuit in the hippocampus. Many recent findings highlight its unique role in hippocampal function, however (Brun et al., 2002; Remondes and Schuman, 2004; Nakashiba et al., 2008). Some interesting features of this pathway include its topographic projection pattern and its sensitivity to neuromodulators.
A topographic projection in which LECor MEC-derived axons terminate in different regions allows the EC to send nonspatial and spatial information to distinct neuronal populations in area CA1 (Steward, 1976; Witter and Amaral, 2004). The efferents from area CA1 are also topographically organized such that neurons in proximal CA1 send projections back to the MEC, while neurons in distal CA1 project back to the LEC (Tamamaki and Nojyo, 1995). Thus, two independent circuit loops for nonspatial and spatial information exist between the EC and CA1. This architecture, based on the TA pathway, may allow the hippocampus to independently process nonspatial and spatial information, providing an unique role for the TA pathway in the hippocampus.
Sensitivity to neuromodulators is another feature of the TA pathway. In recordings from control mouse hippocampal slices, DA induces a larger depression at proximal compared to distal TA-CA1 synapses. However, hippocampal slices prepared from offspring of poly(I:C)-treated mothers exhibit a significantly larger DA-induced depression, selectively at distal TA-CA1 synapses compared to controls, which may

abolish the differential control of LEC and MEC inputs by DA. Our present observations, based on both electrophysiology and c-Fos expression, suggest that the offspring of poly(I:C)-treated mothers have altered DA-mediated control of the TA pathway and may have an abnormality in object information processing. This altered DA signaling may be due to the abnormal development of the DA system recently observed in MIA offspring (Vuillermot et al., 2010), although the hippocampus and EC in this animal model have not yet been examined for expression of DA receptor or transporter with regard to the TA pathway. It is worth noting that the antipsychotic drug, clozapine, effectively blocks DA-induced depression at TA-CA1 synapses (Otmakhova and Lisman, 1999), indicating that the TA pathway may be a locus for clozapine action in schizophrenia.

The proper integration between Schaffer-collateraland TA-CA1 synapses is critical for controlling spike initiation and synaptic plasticity in CA1 pyramidal neurons (Dvorak-Carbone and Schuman, 1999; Remondes and Schuman, 2002; Jarsky et al., 2005; Ang et al., 2005; Dudman et al., 2007). Thus, abnormal DA-mediated control of TA-CA1 synapses, together with reduced number of excitatory inputs on CA1 pyramidal neurons, will likely lead to altered synaptic plasticity or information integration in CA1 pyramidal neurons in the MIA offspring.

Perseveration behavior and hypersensitivity to a novel object

Our behavioral analyses indicate that the offspring of poly(I:C)treated mothers show behavioral inflexibility in the Morris water maze task and increased sensitivity in the novel object recognition task. The normal acquisition of an initial platform location in the water maze task indicates that MIA offspring have normal spatial navigation, such as

in self-localization, route learning and motor function for swimming (Redish and Touretzky, 1998). A selective deficit in learning a moved platform location suggests that these animals have difficulty in adapting to a modification introduced in previously acquired information, which may correspond to perseveration behavior. Such behavior is defined as contextually inappropriate and unintentional repetition, and is often observed in schizophrenia patients (Crider, 1997). These patients have difficulty in switching behavioral-strategy, or reversal learning, and tend to repeat the same response or strategy (Fey, 1951; Nolan, 1974; Floresco et al., 2008). Interestingly, a recent human study indicates that maternal infection is correlated in the offspring with impaired performance in the Wisconsin card sorting test, which provides a good measure of perseveration behavior (Brown et al., 2009). That is, the subset of schizophrenia subjects born to infected mothers display a more severe behavioral deficit in this test than schizophrenia subjects born to non-infected mothers. In experimental animals, the offspring of poly(I:C)-treated mothers display deficits in reversal learning in a left–right discrimination task (Meyer et al., 2006). Perseveration behavior is also observed in hippocampus-lesioned animals in the Morris water maze task (Whishaw and Tomie, 1997). Both hippocampus-lesioned animals (Kim and Frank, 2009) and rodents injected with a DA receptor agonist (Boulougouris et al., 2009) exhibit impaired reversal learning. Thus, abnormalities in the hippocampus and the DA system may play a key role in perseveration behavior.

In light of perseveration behavior in the water maze, the increased sensitivity to a novel object that we observe in the MIA offspring may be due to an enhanced expectation for the original object, which will increase novelty for a switched object.

Importantly, the abnormal preference in the MIA offspring was observed selectively in the novel object recognition (nonspatial information), but not in the object location (spatial information), task.

These altered behaviors in the MIA offspring may be due to abnormal processing of nonspatial information in the hippocampus, as observed in our slice physiology and c-Fos expression analyses. Contrary to our results, Ozawa et al. (2006) and Ibi et al. (2009) reported a deficit in a novel object recognition test. However, they used a more severe regimen of poly(I:C) treatment for 5–6 consecutive days at a different time during development, and they employed a longer time away from the object, making the test more of a memory test than a novelty preference test. Moreover, Golan et al. (2005) obtained a result similar to ours in a novel object recognition test using maternal injection of lipopolysaccharide to mimic maternal bacterial infection. It should also be noted that, in the water maze test, Zuckerman and Weiner (2005) reported some similarities and some differences in results compared to ours. However, they used rats and administered poly(I:C) at a much later time in gestation.

Integration of information processed in parallel

A major feature of brain function is parallel information processing. For example, visual information is processed in two distinct information streams: a ventral stream that subserves object recognition, or ‘‘what” perception, and a dorsal stream that primarily subserves spatial information, or ‘‘where” perception (Ungerleider and Haxby, 1994). The distributed information, processed in different brain areas, must be integrated for coherent perception.

Recent imaging and physiology studies report abnormal visual object recognition in schizophrenia patients (Doniger et al., 2002; Wynn et al., 2008). Wynn et al. (2008) measured activity in early retinotopically organized areas (V1–V4), motion-sensitive areas (human area MT) and object-recognition areas (lateral occipital complex), and found that schizophrenia patients display more widely-distributed activation in areas involved in object recognition than controls. Thus, the abnormal distribution of activity in object-selective cortex in schizophrenia patients may indicate a problem in the integration of spatial and nonspatial information.

In the hippocampus, the integration of spatial and nonspatial information is critical for constructing a neural representation of environmental context. As such, the hippocampus plays an essential role in contextual memory formation. Interestingly, several studies indicate that schizophrenia patients have a severe problem in contextual memory formation (Boyer et al., 2007; Rizzo et al., 1996; Danion et al., 1999), although other types of memory, which do not require contextual information, are relatively intact. Our findings raise the question of whether this memory deficit could be due to abnormal DA-mediated control of the TA pathway in the hippocampus. Interestingly, a recent study using model simulation predicts a dominance of object over spatial information processing in the medial temporal lobe of schizophrenia patients (Talamini and Meeter, 2009). Thus, the altered hippocampal information processing we observe in MIA offspring may underlie some schizophrenia-like behaviors of these animals, such as in pre-pulse inhibition, latent inhibition and perseverative behavior. Further investigation based on this perspective may shed new light on the pathophysiology of schizophrenia.

Methods

Animals

Pregnant C57BL/6J mice were injected either i.v. with 5 mg/kg or i.p. with 20 mg/kg poly(I:C) potassium salt freshly dissolved in 0.9% sterile saline on E12.5. Both doses and routes of administration have been used to induce MIA and behaviors relevant to schizophrenia in adult offspring (Meyer et al., 2006; Smith et al., 2007). Control females were injected with the same volume of saline. The offspring were undisturbed until weaning on P21. Offspring were behaviorally tested from 6 to 11 weeks for pre-pulse inhibition (PPI), latent inhibition and open field exploration, to confirm behavioral deficits observed in previous studies (Shi et al., 2003; Smith et al., 2007; data not shown), and for Morris water maze, object location and novel object recognition. Five litters each from salineand poly(I:C)-treated mothers were generated (3 by i.p. injection and 2 by i.v. injection), and four litters each were used for analysis in the present study. One pair of litters generated by i.p injection was not included because control and MIA offspring did not show significant differences in PPI and latent inhibition. The litters generated by i.p. injection (24 offspring pairs in total) were used for the analysis in Figs. 1A and B, 2D and E and for all of the behavioral analysis in Fig. 5. The rest of experiments, including those in supplemental figures, were conducted using the litters generated by i.v. injection (28 offspring pairs in total). No striking differences in PPI, latent inhibition or open field behavior was observed between the offspring of i.p. and i.v. injected mothers. To avoid influences of prior behavioral testing, animals used for electrophysiology, immunohistochemisty and c-Fos expression analysis were sacrificed at least 3 days after the last behavioral test.

Hippocampal slice preparation

For each experiment, hippocampal slices were made from paired adult offspring (same sex and age, between 7to 15week-old, randomly selected from each litter for each set of experiments without regard to the results of behavioral tests) from salineand poly(I:C)-treated mothers. In brief, a vibrating microtome (Leica VT1000S) was used to cut transverse hippocampal sections from the intermediate part of a dorsoventral axis of the hippocampus (400 lm thickness) in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 2.5 KCl, 1.3 MgSO4, 2.5 CaCl, 1.0 NaH2PO4, 26.2 NaHCO3, 11.0 glucose. Slices were recovered at room temperature for at least 2 h in an interface chamber, and then transferred to a submerged recording chamber perfused with ACSF. Concentric bipolar tungsten electrodes (FHC) and stimulus isolators (Axon Instruments) were used for the stimulation.

Electrophysiology

Extracellular field potential recordings were made with 1–3 MX resistance microelectrodes filled with 3 M NaCl using a bridge amplifier (Axoclamp 2B, Molecular Devices). The recordings were made at 25 °C. Whole-cell voltage-clamp recordings from CA1 pyramidal neurons were obtained without visualization with an Axopatch 200B (Molecular Devices). The internal solution of whole-cell patch pipettes contained (in mM) 115 cesium gluconate, 20 cesium chloride, 10 sodium phosphocreatine, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.3 NaGTP (pH 7.3). The membrane capacitance was canceled and series resistance was compensated (60– 70%) for paired-pulse facilitation

experiments, but uncompensated for miniature recordings. Recordings were discarded when the series resistance was over 20 MX or either series or membrane resistance changed more than 20% (30% for mIPSC recordings) during data acquisition. The amplitude of test pulses was 20–40 lA for recordings from SC-CA1 synapses and 50–150 lA for recordings from TA-CA1 synapses, and the duration of pulse was 100 ls. The test pulses were applied once every 30 s. Dopamine was obtained from Sigma. All other drugs were obtained from Tocris. For mEPSC recordings, whole-cell patch clamp recordings were obtained from CA1 pyramidal neurons in extracellular solution containing TTX (1 lM) and bicuculline (10 lM) at 25 °C. For mIPSC recordings, the internal solution of patch pipettes was a cesium chloride-based solution (in mM): 115 cesium chloride, 20 KCl, 10 sodium phosphocreatine, 10 HEPES, 0.2 EGTA, 2 MgATP, 0.3 NaGTP (pH 7.3). The recordings were made at 28°C, using TTX (1lM), NBQX (20 lM) and APV (50 lM) to block excitatory synaptic transmission, and the extracellular potassium concentration was increased from 2.5 mM to 5 mM to enhance the frequency of miniature synaptic events. Membrane voltage was clamped at 70 mV. For the analysis of paired-pulse facilitation, whole-cell patch clamp recordings were obtained from CA1 pyramidal neurons. The membrane potential was clamped at 60 mV, and recordings were made at 25 °C under bicuculline (10 lM) and CGP55845 (1 lM) to block inhibitory synaptic transmission. The interstimulus interval was 50 ms. The LTP induction protocol was a single train of 100 pulse stimuli at 100 Hz. All stimulus pulses were of the same length and amplitude as test pulses. The baseline fEPSP slope prior to LTP induction was adjusted within the range of 0.2– 0.3 mV/ms.

Behavioral manipulations for c-Fos expression analysis

All the behavioral manipulations were carried out at night (12– 4 Am) to maximize active exploration of the environment. The objects used for novel object exposure were two small children's toys, made of either plastic or wood. The new cage for novel place exposure was in the same color and dimensions as the original cage, but had new woodchip bedding of a different scent and texture than the prior bedding, and the new cage did not have a food box on the ceiling. Such sensory cue changes in the environmental

context, without geometric changes in the cage configuration, are sufficient for remapping of hippocampal place cell activities (Anderson and Jeffery, 2003), implying that animals should recognize the new cage as a different context. More radical changes in the new cage configuration were not made in an effort not to introduce fear or anxiety in the mice.

Behavioral tests

Water maze task

Water maze testing followed previously published procedures (Zuckerman and Weiner, 2005). Briefly, mice were introduced to the maze (water made opaque with non-toxic latex paint) in a random spot and allowed 60 s to find a platform submerged 1 cm under the water. The walls of the room contained visual cues (four 0.5 m diameter pieces of different color paper cut into different shapes). Mice were given four trials per session, two sessions per day separated by approx 4 h. In between trials mice were allowed to recover for approx 5 min in a cage lined with paper towels and warmed with an electric

heating pad. If the mice found the platform within 60 s, the time to find the platform was recorded and the mouse was removed after 10 s on the platform; if it did not find the platform it was placed on the platform for 10 s and the time was recoded as 60 s. On the afternoon of the 4th day, the platform was removed and swim pattern was recorded for 60 s (probe trial). On the day following the probe trial, the platform was placed in the opposite quadrant of the pool, and three more sessions were conducted with the platform in the novel location. Finally, the water level was dropped and four visible platform trials were conducted.

Novel object recognition and object location recognition tasks

Mice were first allowed to explore the environment (a 50 cmsquare white plastic box) for 10 min. They were then removed for 5 min, during which time two different objects were placed in opposite corners of the box, and the mice were allowed to explore the objects for 5 min. The mice were again removed from the box for 5 min, and for the novel object recognition test, one of the objects was replaced with a novel object (the target object), and the mice were allowed to explore for another 5 min. On the following day, the object location test was administered. The procedure was identical to the previous one except that new objects were used, and rather than replacing the target object during the second phase of the test, it was moved 90 degrees to a different corner of the box (see Fig. 5D). To eliminate experimental bias due to innate preference for an object, the five objects used (a plastic pickle, a ‘‘minikoosh” ball, a plastic top, a small metal knife, and a small candle) were pseudo-randomized such that different mice saw different combinations of objects at each stage of the test; however no innate preferences

for an object were observed.

Data analysis

Electrophysiology

Data were collected using a custom-written program (LabView data acquisition system; National Instruments) for extracellular recordings, or DigiData 1200 and pClamp 9 (Molecular Devices) for whole-cell recordings. For the analysis of miniature synaptic transmission, current traces were acquired for 3 min, and mEPSCs or mIPSCs were detected using a template-matching algorithm of Clampfit 9 (Molecular Devices). All numerical values listed represent mean ± SEM. Student's t-test was performed to analyze the significance of the data.

Immunohistochemistry

Slices (400lm thickness) were prepared from randomly selected pairs of animals using the same procedures as for electrophysiology recordings. After cutting, slices were quickly fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for at least 2 days. Thin (50 lm) sections were cut with a vibrating microtome (Leica VT1000S). The sections were incubated overnight with either of 1:250 concentration of anti-c-Fos (sc-52) (Santa Cruz), 1:1000 of anti-synaptophysin (Millipore), 1:1000 of anti-synapsin I (Millipore), 1:1000 of anti-Bassoon (Stressgen), 1:1000 of NeuN (Millipore), 1:1000 of GluR1 (Millipore), or 1:1000 of anti-MAP2 (Sigma) antibodies. The incubation was carried out at room temperature in Tris-buffered saline containing 0.2% Triton X-100, BSA 2%, NGS 4%, followed by 4 h of secondary-antibody incubation with 1:1000 of

Alexa 488-conjugated anti-rabbit and 1:1000 of Alexa 543-conjugated anti-mouse antibodies (Invitrogen). For the analysis of immunohistochemistry experiments, images were obtained with Zeiss LSM 510 laser scanning confocal microscopes using a Plan-Neofluor 10 /0.3 air objective. Alexa 488 and 546 were visualized by excitation with the 488 line of an argon ion laser and the 543 nm line of a HeNe laser, respectively. The optical section was 20 lm and fluorescent signals were acquired throughout the section thickness. Each 50 lm section was obtained from a different 400 lm slice and two sections were analyzed from each animal. Slices were obtained from the same septo-temporal position in all experiments. To count the number of c-Fos positive neurons, fluorescent signals of less than the mean + two times the standard deviation were excluded. Then automated particle analysis was carried out using ImageJ (NIH) based on the following criteria: the particle size was larger than 39 lm2 and the circularity was larger than 0.5. Statistical differences between animals groups were assessed by ANOVA.

Behavioral analysis

Water maze task

All trials were recorded by a camera suspended over the pool, and data was analyzed with Ethovision (Noldus). Swim speed, mean distance swam and performance in the visible platform task was not significantly different between groups (data not shown).

Novel object recognition and object location tasks

The trials were recorded by an overhead camera, and object investigation was

measured as the number of times a mouse brought its nose within 2 cm of the object, by an observer blind to the identification of each mouse. Data was expressed as percent nose pokes to the target object [nose-pokes to target]/[nose-pokes to target + nose-pokes to non-target object] 100), where the target object is represented by the novel object during the novel object recognition task and by the re-located object during the object location task.

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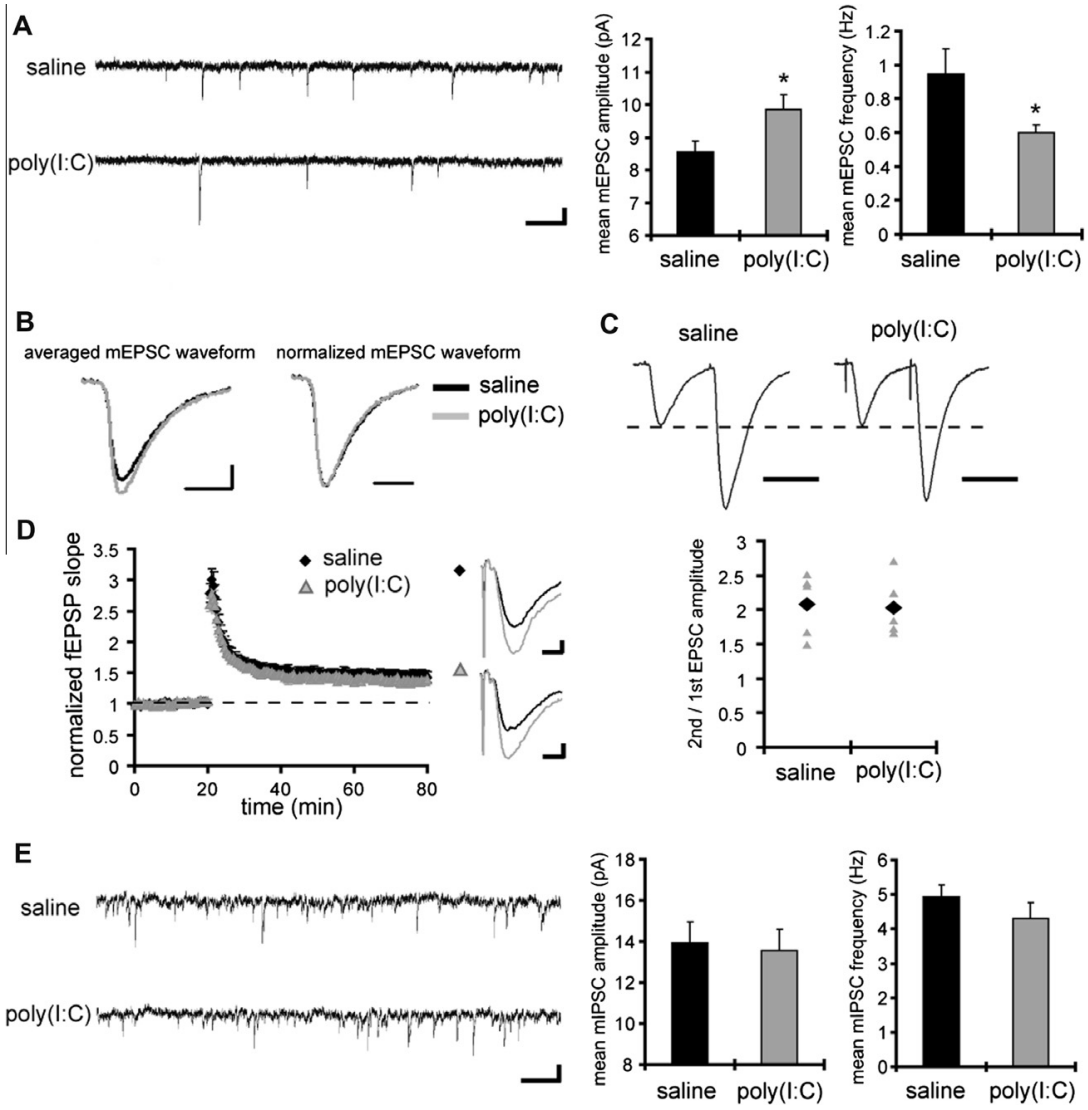

Fig. 1. CA1 pyramidal neurons in MIA offspring display a reduced frequency and an increased amplitude of mEPSCs, but no significant difference in mIPSCs. (A) Typical recordings and quantification of mEPSCs from CA1 pyramidal neurons are shown (saline: n = 11 neurons, poly(I:C): n = 12 neurons, 4 pairs of animals) (scale bar = 500 ms, 10 pA) (*$p < 0.05$ relative to control). (B) Averaged mEPSC waveforms from animals used for the analysis in A are shown on the left. The mEPSC waveform in MIA offspring shows larger amplitude compared to that in control animals. To examine the kinetics of the waveforms, the amplitudes of mEPSC waveforms are normalized on the

right. There is no significant difference in kinetics of mEPSCs between the groups. (C) Whole-cell voltage-clamp recordings were performed in CA1 pyramidal neurons under inhibitory blockade with the GABA receptor antagonists bicuculline (10 lM) and CGP55845 (1 lM). Membrane potential was clamped at 60 mV. Paired-pulse facilitation (PPF) was analyzed at Schaffer-collateral-CA1 synapses. The top panel shows representative waveforms (interstimulus interval is 50 ms; scale bar = 50 ms), and the bottom panel shows the PPF ratio (2nd/1st EPSC amplitude) of individual recordings (gray triangles), and the mean values (black diamonds) (n = 5 neurons for each group, 3 pairs of animals). (D) Extracellular field recordings were performed at Schaffer-collateral-CA1 synapses under fast inhibitory transmission block with the GABAA receptor antagonist bicuculline. The left panel shows the normalized fEPSP slope from control animals (black diamonds) and MIA offspring (gray triangles) and LTP was induced at 20 min by a single train of 100 stimuli at 100 Hz. The right panel shows representative waveforms before (black) and after (gray) LTP induction (scale bar = 0.2 mV, 5 ms). No significant difference is observed in LTP magnitude between animal groups (n = 8 slices for each group, 3 pairs of animals). (E) Typical recordings and quantification of mIPSCs from CA1 pyramidal neurons are shown (n = 12 neurons for each group, 3 pairs of animals). A cesium chloride-based solution was used as the internal solution of patch pipettes and recordings were made at 28 °C in the presence of TTX (1 lM), NBQX (20 lM) and APV (50 lM) to block excitatory synaptic transmission. The extracellular potassium concentration was increased from 2.5 to 5 mM to enhance the frequency of miniature synaptic events. No significant difference is observed in mIPSC amplitude or frequency between the groups.

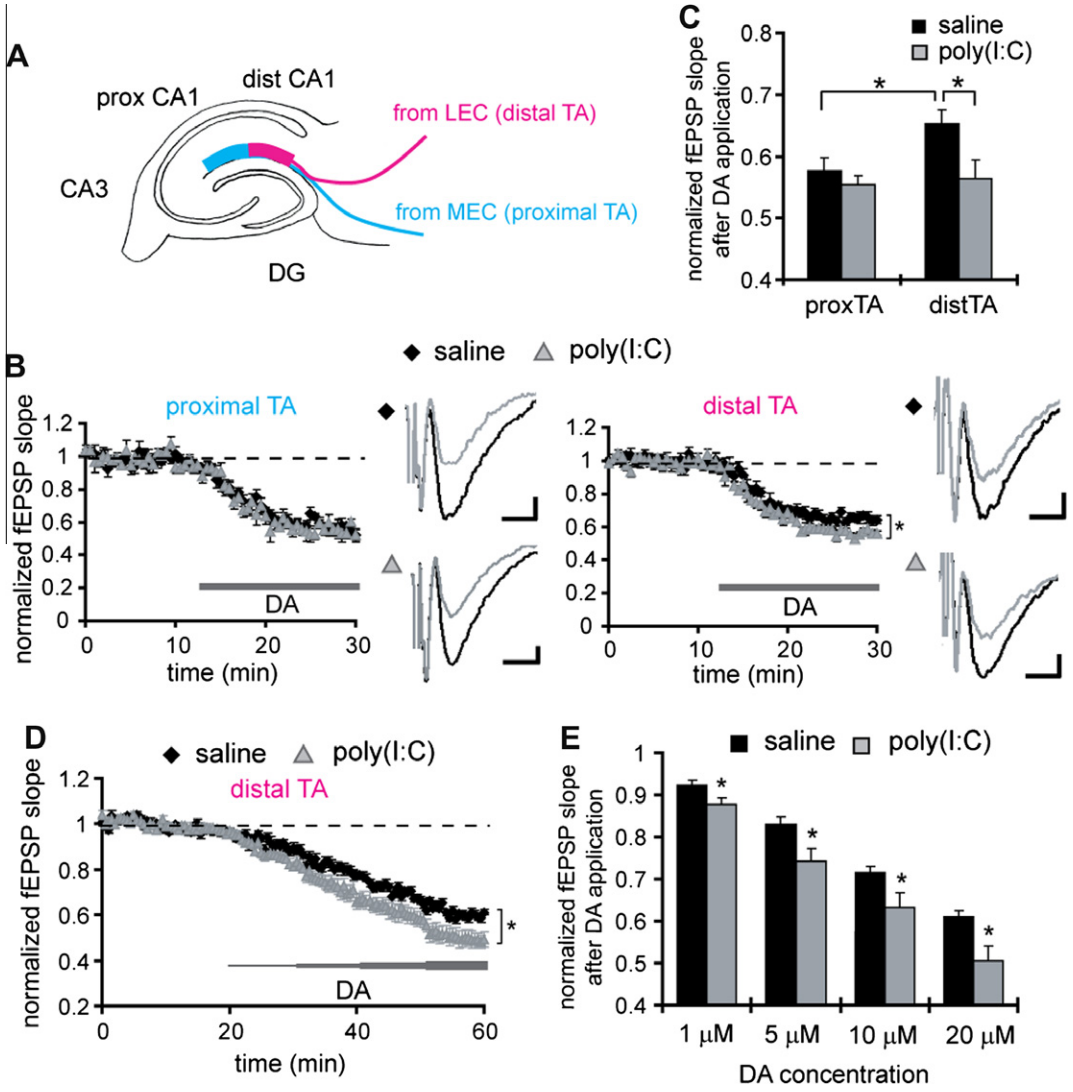

Fig. 2. The offspring of poly(I:C)-treated mothers display increased DA-induced depression at distal temporoammonic-CA1 synapses. (A) A schematic diagram is shown depicting the two distinct axonal projections in the TA pathway from MEC and LEC. (B) Extracellular field recordings are obtained simultaneously from proximal and distal TA-CA1 synapses. For each recording from proximal or distal TA-CA1 synapses, the left panels show the normalized fEPSP slopes from control (black diamonds) and MIA offspring (gray triangles), and the right panels show representative traces of fEPSP

waveforms before (black) and after (gray) DA application (scale bar = 0.05 mV, 5 ms). The test pulse was applied every 30 s and DA (20 lM) was bath applied during the time indicated by the thick line (*p < 0.05 relative to control) (saline: n = 7 slices, poly(I:C): n = 6 slices, 4 pairs of animals). (C) In hippocampal slices prepared from control animals, DA induces a significantly larger depression at proximal compared to distal TA-CA1 synapses. While the slices prepared from MIA offspring show a normal DA-induced depression at proximal synapses, they exhibit a significantly larger depression at distal TA-CA1 synapses, compared to controls (*p < 0.05). (D) Hippocampal slices prepared from MIA offspring show increased DA-induced depression at distal TA-CA1 synapses. The DA concentration was increased every 10 min, from 1 to 20 lM (*p < 0.05 relative to control). (E) Analysis of the data in D is shown. The mean fEPSP slopes at 5–10 min after the application of each designated concentration of DA were analyzed. Slices prepared from MIA offspring show a significantly larger depression at each DA concentration examined (n = 12 slices for each group, 8 pairs of animals) (*p < 0.05 relative to control).

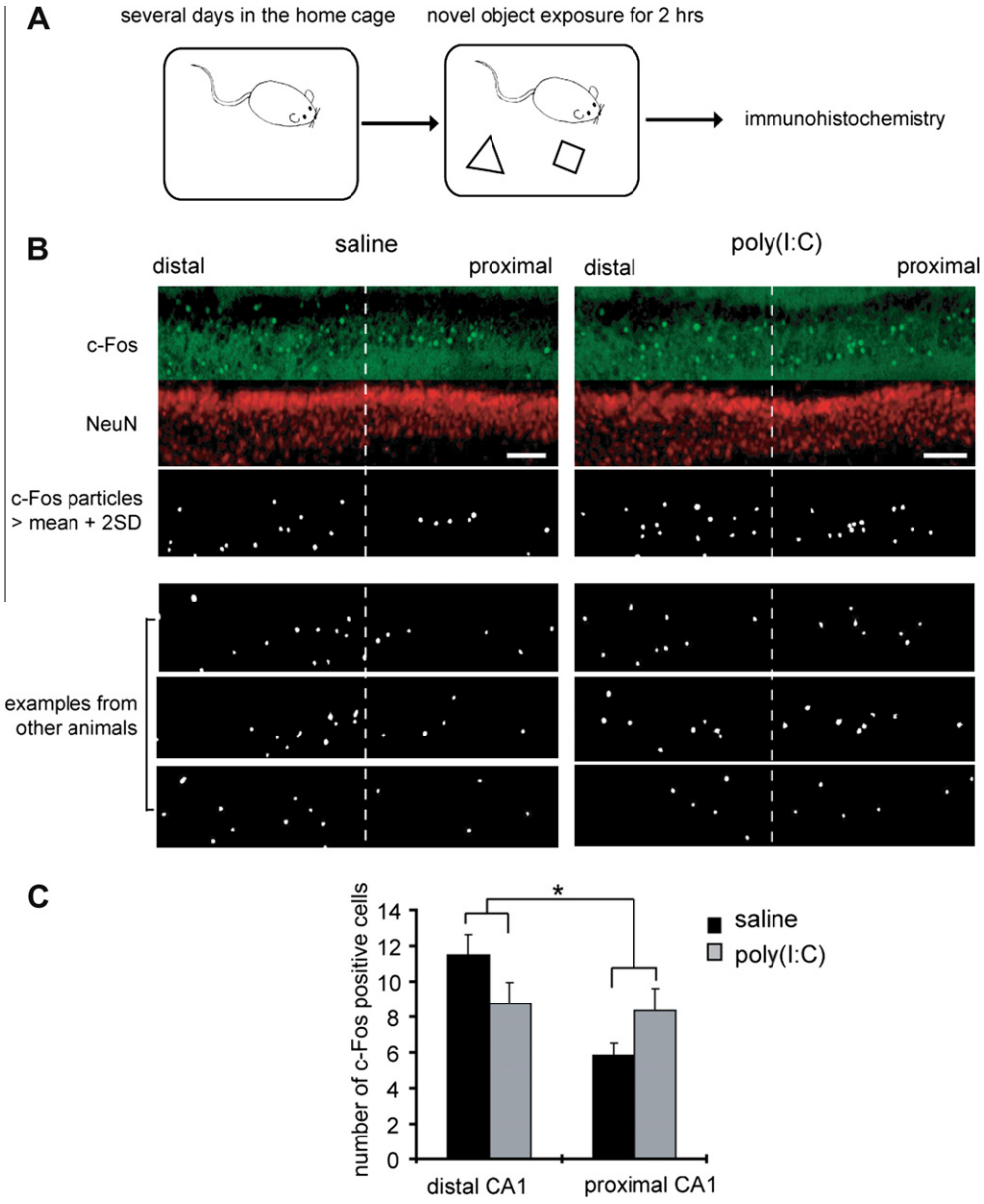

Fig. 3. The offspring of poly(I:C)-treated mothers display abnormal c-Fos expression in area CA1 pyramidal neurons following novel object exposure. (A) A schematic diagram of the behavioral procedure is shown. After mice were accommodated to their new home cages for a few days, two novel objects were placed in the cage. After a 2 h exposure to allow c-Fos gene activation to be expressed as protein, the hippocampus was processed for immunohistochemistry. (B) Examples of c-Fos expression in the

pyramidal layer of area CA1 are shown. The pyramidal layer was divided into equal proximal and distal regions. The c-Fos signals that are larger than the mean + two times the standard deviation, were quantified using ImageJ (see Section 2 for details) (scale bar = 100 lm). (C) The number of c-Fos signals was quantified in proximal and distal regions of the CA1 pyramidal layer. A two-way ANOVA was performed with 2 variables: animal group (saline vs. poly(I:C)) and CA1 subregion (distal vs. proximal), and revealed a significant interaction (*$p < 0.05$) (n = 6 pairs of animals, 2 sections analyzed and averaged from each).

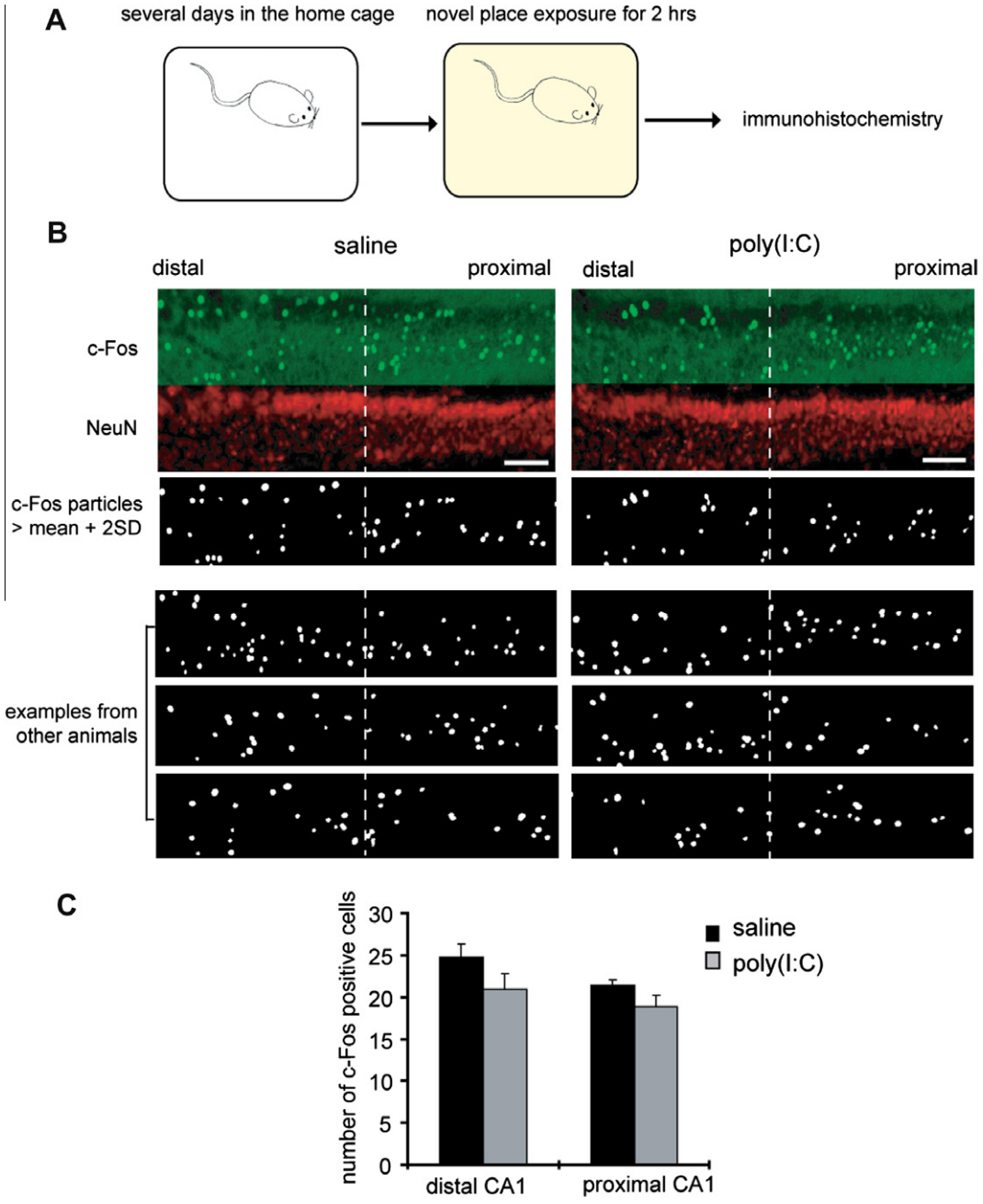

Fig. 4. The offspring of poly(I:C)-treated mothers display comparable c-Fos expression in area CA1 pyramidal neurons following novel place exposure. (A) A schematic diagram of the behavioral procedure is shown. After mice were accommodated to the new home cage for a few days, they were moved to a novel cage. After a 2 h

exposure, immunohistochemistry on the hippocampus was carried out. (B) Examples of c-Fos expression in the pyramidal layer of area CA1 are shown. The pyramidal layer was divided into equal proximal and distal regions. The c-Fos signals that are larger than the mean + two times the standard deviation, were quantified using ImageJ (see Section 2 for details) (scale bar = 100 lm). (C) The number of c-Fos signals was quantified in proximal and distal regions of CA1 pyramidal layer (n = 5 pairs of animals, 2 sections analyzed from each).

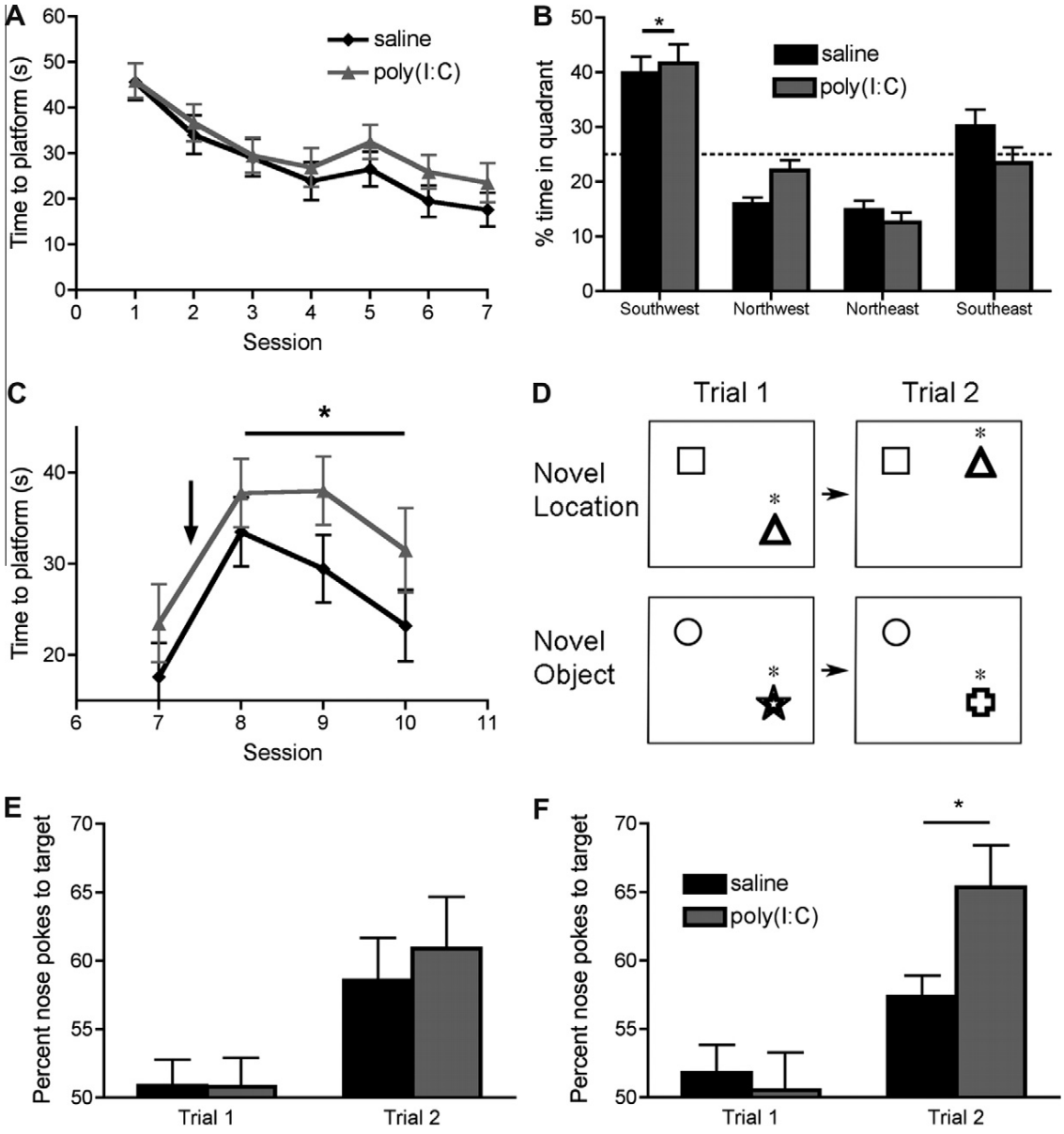

Fig. 5. The offspring of poly(I:C)-treated mothers display behavioral inflexibility in platform relocation in the Morris water maze task, and abnormal preference in the novel object recognition task. (A) In the Morris water maze, the latency to find the platform is similar in the MIA offspring compared to controls (saline: n = 16, poly(I:C): n = 17). (B) Both control and MIA offspring show a significant learned preference for the target quadrant in the session 7 probe trial, which was not present before training (*$p < 0.05$ vs. all other quadrants). (C) When the location of the platform was moved after

training (indicated by arrow), the MIA offspring do not learn the new location as quickly as controls (*$p < 0.05$). The experimental groups diverge significantly after the platform is moved, at the point indicated by the arrow. (D) A graphical representation of the object location and novel object recognition tests illustrates how the location of the target object, or the type of target object itself, is changed in the 5 min interval between trial 1 and 2. Asterisks indicate the target objects. (E) In the novel location test, both control and MIA offspring show a significant preference for the target object in trial 2, compared to trial 1 ($p < 0.05$), but there is no difference between control and MIA offspring. (F) In the novel object recognition test, both groups also display a significant preference for the target object in trial 2 compared to trial 1 ($p < 0.05$). Moreover, compared to control animals, the MIA offspring show a significantly greater preference for the target object in trial 2 ($n = 16$ animals per group) (*$p < 0.05$).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2010.03.004.

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Chapter 10

Immune involvement in autism spectrum disorder as a basis for animal models

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Abstract

Several of the environmental stimuli suggested to play a role in the pathogenesis of ASD involve altered immune responses during gestation. In this review, we discuss maternal immune activation as a primary risk factor for ASD, with an emphasis on recent findings from animal models of prenatal immune challenges. We further address the presence of autoantibodies as an additional immune-related autism risk factor, drawing upon work done in rodent and monkey models. We then explore the intersection between genetic and environmental susceptibility, with a focus on gene-environment interactions and immune involvement in genetic risk factors for autism. Finally, we provide emerging evidence for the role of immune dysregulation in the pathogenesis of ASD.

Introduction

Autism spectrum disorder (ASD) consists of a heterogeneous group of syndromes defined by the presence and severity of repetitive/stereotypic behaviors, abnormal social interaction and impaired communication. ASD is highly heritable, as indicated by increased concordance in monozygotic versus dizygotic twins and by elevated risk observed in the siblings of an affected child (Rosenberg et al., 2009). Accordingly, genome-wide association and linkage studies have uncovered a number of genetic copy number variants, short nucleotide polymorphisms and common risk variants that increase the susceptibility for ASD (Betancur, 2011). Despite continued advances in next generation sequencing, very few ASD cases can be attributed to a defined genetic etiology, and it is estimated that identified genetic risk factors collectively account for only 10-20% of ASD cases (Abrahams & Geschwind, 2008; Anney et al., 2012). Moreover, findings from two very large autism twin studies demonstrate a significant difference in concordance between dizygotic twins and non-twin siblings, indicating a substantial contribution of the maternal-fetal environment to ASD risk (Hallmayer et al., 2011; Rosenberg et al., 2009). This, combined with profound increases in autism prevalence over the past two decades ("Prevalence of autism spectrum disorders--Autism and Developmental Disabilities Monitoring Network, 14 sites, United States, 2008," 2012), highlights the importance of environmental factors in the etiology of ASD.

Immune-related risk factors for ASD

Maternal immune activation

Several environmental factors increase susceptibility to ASD, with early immune activation being one of the most strongly supported by epidemiological, clinical and animal studies (Brown, 2012). Large epidemiological studies assessing over 10,000 ASD cases from Danish national birth records and over 4,000 ASD cases identified from Swedish residence registries demonstrate that maternal infection during pregnancy increases the risk for ASD (Atladottir et al., 2010; Lee et al., 2012). In addition, early infection with any of a variety of pathogens, including rubella, cytomegalovirus or varicella, is linked to elevated autism risk (Brown, 2012). The impact of maternal immune activation (MIA) on ASD is further supported by associations between maternal fever during pregnancy and ASD (Zerbo et al., 2012). Moreover, elevation of the cytokines IFN-γ, IL-4 or IL-5 in maternal blood and TNFa and MCP-1 in amniotic fluid is linked to risk for ASD (Abdallah et al., 2011a, 2011b, 2012; Goines et al., 2011).

In animals, MIA is induced by perinatal infection with specific pathogens, exposure to microbe-associated molecular patterns (MAMPs) or direct administration of recombinant cytokines. MIA by any of these inflammatory stimuli leads to autismand schizophrenia-related behavioral abnormalities in the offspring (reviewed in (Harvey & Boksa, 2012a; Macedo et al., 2012; Meyer, Feldon, & Fatemi, 2009; Oskvig, Elkahloun, Johnson, Phillips, & Herkenham, 2012)). Intranasal inoculation of pregnant mice with influenza virus, for example, yields offspring with heightened anxiety, deficient sensorimotor gating and reduced social interaction, and a localized reduction in Purkinje cell number (Shi et al., 2009), all features of autism. Moreover, offspring of mothers injected with the viral mimic poly(I:C) during midgestation exhibit the three cardinal behavioral symptoms of autism: impaired sociability and social preference, defective

communication as assessed by pup and adult ultrasonic vocalizations and odorant communication by scent marking, and elevated repetitive behaviors as measured by self-grooming and stereotyped marble burying (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Yee, Schwarting, Fuchs, & Wohr, 2012). Similar changes in core ASD-related behaviors are observed after first trimester injection of LPS in pregnant rats (Baharnoori, Bhardwaj, & Srivastava, 2012). Midgestational injection of recombinant IL-6 in pregnant mice is sufficient to induce behavioral deficits in the offspring that are comparable to those induced by maternal influenza or poly(I:C) injection (Hsiao & Patterson, 2011; Smith, Li, Garbett, Mirnics, & Patterson, 2007). Similarly, maternal IL-2 injection leads to anxiety-like and repetitive behaviors in mouse offspring (Ponzio, Servatius, Beck, Marzouk, & Kreider, 2007). In contrast, over-expression of the anti-inflammatory cytokine IL-10 in pregnant mice sufficiently prevents MIA-induced behavioral and biochemical abnormalities in the offspring (Meyer et al., 2008). In addition, several studies utilizing early postnatal immune activation in rodents, to better mimic second trimester human brain development, also report long-term changes in neuropathology and behavior (Bland et al., 2010; Harvey & Boksa, 2012b).

The impact of MIA on brain and behavior is further supported by non-human primate studies. Pregnant rhesus monkeys infected with influenza virus during the third trimester yield offspring with significant decreases in cortical gray matter and reduced white matter in the parietal lobe, in addition to decreased social contact with the mother (Short et al., 2010). Notably, these abnormalities occur in the absence of primary viral infection of the fetal compartment. Low-dose maternal LPS injection in monkeys, however, leads to the opposite neural phenotype in which infant offspring display

increased cortical white matter compared to controls, analogous to changes observed in autistic children, and decreased vocalizations during an intruder paradigm, among several other behavioral abnormalities (Willette et al., 2011). First or second trimester maternal poly(I:C) injection of rhesus macaques also leads to autism-related endophenotypes in the offspring, including motor stereotypies, elevated distress and self-soothing behaviors, and a deficit in verbalizations (Bauman et al., 2011). Overall, that MIA can be translated to non-human primate models to induce ASD-related neuropathology and behavioral symptoms further supports the study of MIA in animals as models with face and construct validity for autism.

The symptomatic overlap between different modes of MIA indicates that the production of pro-inflammatory cytokines in response to immune activation is likely a key pathophysiological step in the development of autism-related impairments. It is well established that MIA induces a maternal pro-inflammatory response that is quickly relayed to the maternal-fetal interface. Several pro-inflammatory cytokines, including IL-6, IL-1b and TNFa, are elevated in the placenta and amniotic fluid by one-hour post MIA challenge (Hsiao, McBride, Chow, Mazmanian, & Patterson, 2012; Mandal, Marzouk, Donnelly, & Ponzio, 2011; Meyer et al., 2006). Maternal LPS injection can lead to dramatic changes in placental physiology, including necrosis, infiltration of immune cells and altered perfusion (Carpentier, Dingman, & Palmer, 2011; Girard, Tremblay, Lepage, & Sebire, 2010). Similar changes are observed in placentas from influenza-infected mothers, in addition to widespread effects on the placental transcriptome (Fatemi et al., 2012). Furthermore, placentas from poly(I:C)-injected mothers exhibit increased activation of decidual immune cells and altered endocrine function (Hsiao & Patterson,

2011). Interestingly, placental trophoblast cells are activated in response to MIA, reflecting a direct transfer of the maternal MIA response to fetally-derived placental cells. It is very likely, however, that the extent of placental damage seen in these various models depends on the dose of the agent administered. Altogether, the downstream effects in the placenta raise the intriguing question of how changes at the maternal-fetal interface impact fetal brain development. Notably, subclassification of ASD cases by behavioral symptoms, patient and family history and biological endophenotypes reveals that immune dysregulation in autism and obstetric complications in the mother cluster together as central characteristics of a subgroup of ASD individuals (Sacco et al., 2012). Moreover, trophoblast inclusion histopathology is found in placentas from births that yield an ASD outcome (Anderson, Jacobs-Stannard, Chawarska, Volkmar, & Kliman, 2007). Given that MIA can induce striking placental pathology, it seems likely that the maternal infection risk factor contributes to pre-term birth, low birth weight and obstetric complications, which are associated with increased risk for ASD (Kuban et al., 2009; Lyall, Pauls, Spiegelman, Ascherio, & Santangelo, 2012; Movsas & Paneth, 2012; Pinto-Martin et al., 2011).

In addition to altering placental status, MIA quickly leads to altered gene expression and cytokine profiles in the fetal brain. Shortly after MIA, the fetal brain exhibits elevated levels of pro-inflammatory cytokines (Arrode-Bruses & Bruses, 2012), suggesting a possible feed-forward propagation of pro-inflammatory MIA responses from the maternal circulation to the placenta, and to the fetus itself. The initial induction of fetal brain cytokines typically diminishes after 24 hours post MIA, but may trigger molecular events that lead to lasting neuroimmune changes. Consistent with this notion,

poly(I:C) offspring exhibit dynamic ageand region-specific changes in brain cytokines throughout postnatal development (Garay, Hsiao, Patterson, & McAllister, 2012; Pacheco-Lopez, Giovanoli, Langhans, & Meyer, 2011). In response to maternal LPS, fetal brains exhibit altered gene expression profiles, with upregulation of genes related to oxidative stress and downregulation of genes related to GABAergic interneuron migration (Oskvig et al., 2012). After maternal influenza infection, embryos exhibit altered brain expression of many genes relevant to autism and schizophrenia, including *Sema3a*, *Foxp2* and *Vldlr* (Fatemi et al., 2008). Furthermore, MIA induction by influenza, poly(I:C) or recombinant IL-6 injection results in distinct as well as shared changes in gene expression in the embryonic brain. Of particular note is the upregulation of genes of the crystallin small heat shock protein family (Garbett, Hsiao, Kalman, Patterson, & Mirnics, 2012). Interestingly, the severity of these gene expression changes correlates with reductions in placental weight, suggesting that placental status can serve as a marker of disrupted neurodevelopment. This is consistent with findings that placental size correlates with the risk for several adult-onset diseases (Barker, Gelow, et al., 2010; Barker, Thornburg, Osmond, Kajantie, & Eriksson, 2010a, 2010b; Eriksson et al., 2012). Acute transcriptional changes and cytokine effects in the fetal brain may serve as an underlying basis for several of the neurodevelopmental impairments observed in MIA offspring (reviewed in (Harvey & Boksa, 2012a; Macedo et al., 2012; Meyer et al., 2009; Oskvig et al., 2012)), including altered cortical neurogenesis (Smith, Elliott, & Anderson, 2012; Soumiya, Fukumitsu, & Furukawa, 2011), hippocampal synaptic transmission (Escobar et al., 2011; Ito, Smith, Hsiao, & Patterson, 2010) and serotonergic and

dopaminergic signaling (Vuillermot, Weber, Feldon, & Meyer, 2010; Wang, Yan, Lo, Carvey, & Ling, 2009; Winter et al., 2009).

Research conducted in MIA animal models collectively demonstrates that transient immune activation is sufficient to cause long-term changes in neurodevelopment and behavior. However, the particular phenotypes induced by MIA differ depending on the type, timing, frequency and route of administration, as well as the genetic and immunological background of the host (Bronson, Ahlbrand, Horn, Kern, & Richtand, 2011; Harvey & Boksa, 2012b; Meyer et al., 2006; Rana, Aavani, & Pittman, 2012). Such variations across MIA studies impedes cross-comparison between findings (Harvey & Boksa, 2012a). At the same time, the information garnered from diverse MIA studies raises the intriguing question of whether differences in the severity, timing and type of MIA can produce distinct forms of neurodevelopmental disorders. The distinction between ASD and schizophrenia is particularly interesting in this regard, as the association between maternal infection and increased schizophrenia risk in the offspring is supported by numerous epidemiological and clinical studies (Brown, 2011, 2012). Moreover, schizophrenia and autism have several, shared clinical features, including social withdrawal, impaired communication and deficient sensorimotor gating, as well as shared genetic susceptibility factors (Talkowski et al., 2012). Offspring of immune activated dams exhibit features of both schizophrenia and autism, including decreased pre-pulse inhibition and social interactions and elevated anxiety leading to the use of MIA in animals to model both disorders (Harvey & Boksa, 2012a; Macedo et al., 2012). It will be interesting to assess how particular autismversus schizophrenia-related symptoms are modulated by changes in timing, intensity and type of MIA (Meyer,

Feldon, & Dammann, 2011). It will also be important to test for endophenotypes that are not shared between these disorders such as repetitive/stereotyped behaviors (Malkova et al., 2012), which are characteristic of autism, and enlarged ventricles (Q. Li et al., 2009) and enhanced sensitivity to hallucinogenic drugs (Moreno et al., 2011), which are characteristic of schizophrenia.

The role of maternal immune activation has been explored in several recent gene x environment studies, supporting the interaction of both environmental influence and genetic susceptibility in the pathogenesis of neurodevelopmental disorders, such as autism and schizophrenia. In transgenic mice expressing mutant human *DISC1*, poly(I:C)-induced MIA interacts with genetic risk to alter social behavior and produce depression and enhance anxiety-like symptoms, along with several neurochemical and neuropathological changes such as decreased number of hippocamal granule cell dendrites and reduced serotonergic neurotransmission (Abazyan et al., 2010). MIA also synergizes with genetic *Nurr1* deficiency to exacerbate sensorimotor and attentional behavior, and to alter expression of dopaminergic markers in the prefrontal cortex and ventral striatum (Vuillermot et al., 2012). In a mouse model of tuberous sclerosis, maternal poly(I:C) injection and *Tsc2* haploinsufficiency together lead to increased gestational miscarriage and abnormal social approach behavior (Ehninger et al., 2012). Together, these studies support the importance of environmental risk factors in predisposing for neurodevelopmental disease in individuals displaying genetic susceptibility.

Autoantibodies in ASD mothers or individuals

Another immune-mediated risk factor for ASD involves serum immunoglobulins that react against self-antigens. Such "autoantibodies" have been identified in plasma from mothers of ASD children and from ASD individuals themselves, and some have been shown to react against neural components, including myelin basic protein and GAD65 in cerebellar Purkinje cells (Braunschweig & Van de Water, 2012; Mostafa & Al-Ayadhi, 2011; Rout, Mungan, & Dhossche, 2012). Although the majority of these autoantibodies are also detected at some frequency in non-ASD, typical controls, particular maternal autoantibodies that react against fetal brain proteins at approximately 37 kDa and 73 kDa display high specificity to autism cases, with striking reproducibility across large experimental cohorts (Braunschweig & Van de Water, 2012). Importantly, autoantibodies with the same reactivity have also been identified in plasma collected during the gestational period from ASD mothers, in contrast to typical studies that isolate the autoantibodies from samples collected up to 18 years post-partum (Croen et al., 2008). Moreover, the autoantibodies are reported to correlate with impaired expressive language in ASD children (Braunschweig et al., 2012). Identifying the specific target antigens to which these autoantibodies react will be critical in uncovering a mechanistic link between maternal autoantibodies and ASD. Furthermore, whether maternal autoantibodies can be detected in corresponding ASD children is still unclear.

Studies that translate this autoantibody risk factor to animal models for ASD are intriguing, but few. An early case study identified anti-Purkinje cell antibodies in a mother of an ASD child (Dalton et al., 2003). Daily injection of this serum into pregnant mice from E10 to E17 yields offspring with decreased exploration and impaired motor coordination. In another study, injection of purified IgG pooled from over 60 ASD

mothers into pregnant mice resulted in heightened anxiety, hyperactivity and an age-specific deficit in sociability (Singer et al., 2009). Lastly, an additional study in rhesus macaques revealed that first-trimester injection of pregnant monkeys with IgG pooled from 21 ASD mothers yields offspring that display whole body stereotypies, hyperactivity and increased nonsocial behavior (Martin et al., 2008).

Much remains to be explored in animal models of the autoantibody risk factor for ASD, including the question of whether maternal autoantibodies can cross the placenta and enter the fetal brain at significant levels. And importantly, what are the antigenic targets of particular autoantibody subclasses, and are these antigens specific to the fetal brain? Interestingly, a study examining reactivity of different ASD-associated maternal autoantibodies to various tissues reveals that some react against proteins from adult brain and fetal small intestine. In such cases, it will be important to assess effects of ASD-related IgG on maternal behavior and physiology and to determine whether reactivity of autoantibodies against non-CNS antigens represents an indirect effect of autoantibodies on brain and behavior. Notably, the impact of autoantibodies derived from ASD individuals, rather than their mothers, has not yet been evaluated in animal models. In addition, the cause of ASD-related autoantibody production has been largely unexplored. It will be fascinating to evaluate whether autoantibody production occurs as a result of other ASD-related genetic and environmental risk factors. Two models of particular interest are MIA, which displays evidence of disrupted immunological tolerance at the maternal-fetal interface (Carpentier et al., 2011; Girard et al., 2010), and MET-deficient mice, in light of findings linking the MET common risk variant to ASD-related autoantibodies in clinical cohorts (Heuer, Braunschweig, Ashwood, Van de Water, &

Campbell, 2011). Finally, autoantibody animal models will serve as a useful tool to explore potential therapeutics for treatment of autism in well-defined subsets of ASD individuals.

Immune dysregulation in ASD

MIA and maternal autoantibody production represent two immune-mediated risk factors whose influences during early life may contribute to ASD onset. In addition to these prenatal immune insults, increasing evidence highlights a role for postnatal immune alterations in the pathogenesis of ASD (Michel, Schmidt, & Mirnics, 2012; Onore, Careaga, & Ashwood, 2012). Several studies report striking immune dysregulation in the neural, peripheral and enteric immune systems of autistic individuals (Fig. 1). Postmortem brains from ASD patients exhibit elevated activation of microglia and astrocytes, in addition to increased levels of pro-inflammatory cytokines (Morgan et al., 2010; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). Notably, transcriptome analysis of ASD brains reveals altered expression of neuronal-related genes, including ASD susceptibility genes, alongside dysregulated expression of immune-related genes related to inflammation and glial activation (Michel et al., 2012; Voineagu et al., 2011). Altered cytokine profiles are also observed in cerebrospinal fluid and sera collected from living autistic individuals (X. Li et al., 2009; Morgan et al., 2010; Vargas et al., 2005). In the periphery, several subsets of leukocytes isolated from ASD blood display altered function, reflecting altered innate and adaptive immune responses (Onore et al., 2012). Interestingly, significant subsets of autistic children experience

gastrointestinal complications, including increased intestinal permeability, lymphoid hyperplasia, lymphocyte infiltration and altered microbial composition (Adams, Johansen, Powell, Quig, & Rubin, 2011; Buie et al., 2010; Finegold, Downes, & Summanen, 2012). Importantly, a number of these autism-associated immune changes are associated with elevated severity of ASD symptoms (Onore et al., 2012).

Immune effects on behavioral symptoms of ASD

Whether immune abnormalities in the brain, periphery and/or gastrointestinal tract are involved in the development or persistence of ASD symptoms is unclear. However, it is well established that immune status across all of these domains is important for normal brain function and behavior. Cytokines in the brain play a fundamental role in synaptic development, and traditional components of the immune system, such as complement proteins and major histocompatibility complexes, are critical for synaptic pruning, neuronal plasticity and the patterning of neural circuits in the normal brain (Deverman & Patterson, 2009; Garay & McAllister, 2010; Needleman & McAllister, 2012; Shatz, 2009; Stephan, Barres, & Stevens, 2012). In the periphery, cytokine responses to infection are known to stimulate vagal nerve afferents, ultimately leading to altered behavior (Dantzer, 2009). Furthermore, knockout of canonical immune components, such as RAG1 and T cell receptor, leads to impaired cognitive behavior and learning and memory (Brynskikh, Warren, Zhu, & Kipnis, 2008; Cushman, Lo, Huang, Wasserfall, & Petitto, 2003; Kipnis, Cohen, Cardon, Ziv, & Schwartz, 2004). In the gastrointestinal tract, the commensal microbiota plays a critical role in the development and status of the immune system (Hooper, Littman, & Macpherson, 2012). Interestingly, germ-free

animals reared in the absence of any microbial exposure, exhibit abnormalities in nociceptive, motor and anxiety-like behaviors (Amaral et al., 2008; Heijtz et al., 2011). Overall, several lines of evidence suggest that immune alterations could directly contribute to the pathogenesis or maintenance of ASD symptoms.

Recent studies in animal models have explored the influence of systemic immune dysfunction on ASD symptoms. In addition to core ASD symptoms and neuropathology, poly(I:C) MIA offspring exhibit lasting peripheral immune dysregulation, including hyper-responsive CD4+ T cells and deficits in splenic regulatory T cells (Hsiao, McBride, Chow, et al., 2012; Mandal et al., 2011). Poly(I:C) offspring also exhibit altered immune function in the mesenteric lymph nodes, as well as deficient intestinal barrier integrity, which is reminiscent of GI abnormalities seen in subsets of ASD individuals (Hsiao, McBride, Chow, et al., 2012; Hsiao, McBride, Hsien, et al., 2012). There is also evidence of microglial activation in some MIA models (Juckel et al., 2011; Roumier et al., 2008). Recent experiments support the notion that systemic immune abnormalities can contribute to the persistence of ASD-related symptoms. Following irradiation and bone marrow transplantation, poly(I:C) offspring exhibit decreased stereotypic and anxiety-like behaviors (Hsiao, McBride, Chow, et al., 2012). Similar findings are observed in a mouse model of Rett syndrome, where irradiation and bone marrow transplantation in MeCP2 knockout mice arrests disease development as measured by lifespan, respiration, body weight and locomotion (Derecki et al., 2012). Importantly, however, the improvement observed in the Rett model is attributed to engraftment of new microglial cells, whereas the improvement observed in the MIA model is primarily an effect on peripheral immunity. In addition, the BTBR strain, which

displays reduced sociability and verbal communication compared to several other mouse strains, also exhibits increased peripheral CD4+ T cells, peripheral B cells and serum and brain immunoglobulin levels, among other immune abnormalities (Heo, Zhang, Gao, Miller, & Lawrence, 2011). The role of immune abnormalities in ASD symptoms in the BTBR mice, however, is unknown. Extending these types of studies to additional animal models for autism will be important for better defining the immune-ASD connection. The notion that immune abnormalities may arise as a result of genetic alterations is also interesting in this regard, in light of the several ASD genes that are relevant to both brain and immune function, including those encoding various HLA haplotypes, receptor tyrosine kinase and complement C4B (Chien et al., 2011; Mostafa & Shehab, 2010; Thanseem et al., 2010; Torres et al., 2006). Unfortunately, few of these immune-related genetic risk factors for autism have been translated to animal models or evaluated for ASD-related immune and behavioral symptoms. Future studies in these areas will be important for identifying converging pathways for several related environmental and genetic risk factors for ASD.

Immune therapies for ASD

Despite numerous findings of immune abnormalities in autism and the known effects of immune modulation on brain and behavior, there are very few published, controlled studies evaluating the efficacy of immunomodulatory therapeutics in treating ASD symptoms (Chez & Guido-Estrada, 2010). Intravenous immunoglobulin treatment has been evaluated in ASD case studies, where up to 10-20% of children undergoing treatment exhibit symptomatic improvement (Gupta, Samra, & Agrawal, 2010). Oral

immunoglobulin treatment, however, has no significant effect on GI symptoms in ASD children (Handen et al., 2009). Also interesting is that fever is associated with improved behavioral symptoms in ASD children (Curran et al., 2007). The anti-inflammatory antibiotic minocycline can rescue synaptic abnormalities and deficits in ultrasonic vocalizations in mouse models of fragile X syndrome, and recent studies report efficacy in treating symptoms in fragile X patients (Bilousova et al., 2009; Hagerman, Lauterborn, Au, & Berry-Kravis, 2012; Rotschafer, Trujillo, Dansie, Ethell, & Razak, 2012). Whether it is also effective in autism is still unclear, although a small pilot study reported no significant clinical improvement after treatment in ASD children (Swedo et al., 2010). The antibiotic D-cycloserine is effective in treating social impairments and stereotypic behavior in animal models relevant to autism (Deutsch et al., 2012; Won et al., 2012) and has also reduced social withdrawal in a small clinical cohort of ASD children (Posey et al., 2004). These effects are commonly attributed to the activity of D-cycloserine as a partial NMDA-receptor agonist, but whether its antibiotic properties are also important in this regard are unknown.

Several other classes of drugs that are used to treat ASD, including antidepressants and antipsychotics, are known to display immunomodulatory properties. The antipsychotic drug risperidone is FDA-approved for treatment of ASD symptoms and also known to modulate immune function, T cell differentiation and serum cytokine profiles via suppression of the AKT/NFkB pathway (Chen et al., 2011; Chen et al., 2012; Kim et al., 2001; Zhang et al., 2004). The antidepressant aripiprazole has effectively treated irritability, hyperactivity and stereotypy in two large randomized controlled trials of ASD children (Ching & Pringsheim, 2012) and is reported to prevent microglial

activation, reduce reactive oxygen species and suppress pro-inflammatory cytokines (Cecchelli, Grassi, & Pallanti, 2010). Fluoxetine, another antidepressent**,** exhibits several effects on immune function (Basterzi et al., 2010; Frick, Rapanelli, Cremaschi, & Genaro, 2009; Nunez et al., 2006; Rogoz, Kubera, Rogoz, Basta-Kaim, & Budziszewska, 2009) and has reduced repetitive behaviors in a double-blind placebo-controlled trial of adults with ASD (Hollander et al., 2012). The beneficial effects of the acetylcholinesterase inhibitors galantamine and donepezil on reducing social withdrawal, irritability and inattention in children with autism (Hardan & Handen, 2002; Nicolson, Craven-Thuss, & Smith, 2006) are believed to be due in part to activation of the cholinergic anti-inflammatory pathway (Pavlov et al., 2009). Finally, the PPARy agonist piaglitozone is known to display immunosuppressive properties and, in a small clinical cohort, significantly improves ASD symptoms, including irritability, lethargy, stereotypy and hyperactivity. (Boris et al., 2007). Additional studies are needed to determine whether the immunomodulatory properties of these drugs are necessary to confer ameliorative effects on ASD symptoms.

Concluding remarks

Increasing evidence points to an immune involvement in the development and/or persistence of ASD symptoms. Maternal immune activation is a principal environmental risk factor for ASD that is sufficient to cause autism-related behavioral abnormalities and neuropathologies in rodent and primate models. Gene x environment studies demonstrate that immune activation in the background of various genetic susceptibility factors can exacerbate pre-existing abnormalities or perpetuate new ASD-associated

endophenotypes. ASD-related autoantibodies represent another immune-mediated pathway affecting neurodevelopment and behavior, but additional studies in animal models are needed to identify the mechanisms underlying autoantibody production and action. ASD individuals also exhibit various aspects of immune dysregulation in the brain, periphery and GI tract. Still unknown are the etiologies and functional consequences of these immune abnormalities on ASD symptoms. Animal models will be useful for determining whether these endophenotypes impact core ASD behaviors and neuropathologies and whether they converge with identified genetic and environmental risk factors for autism. Studies in these areas will sharpen our understanding of immunomodulatory approaches towards treating particular autism-associated endophenotypes in defined subclasses of ASD individuals.

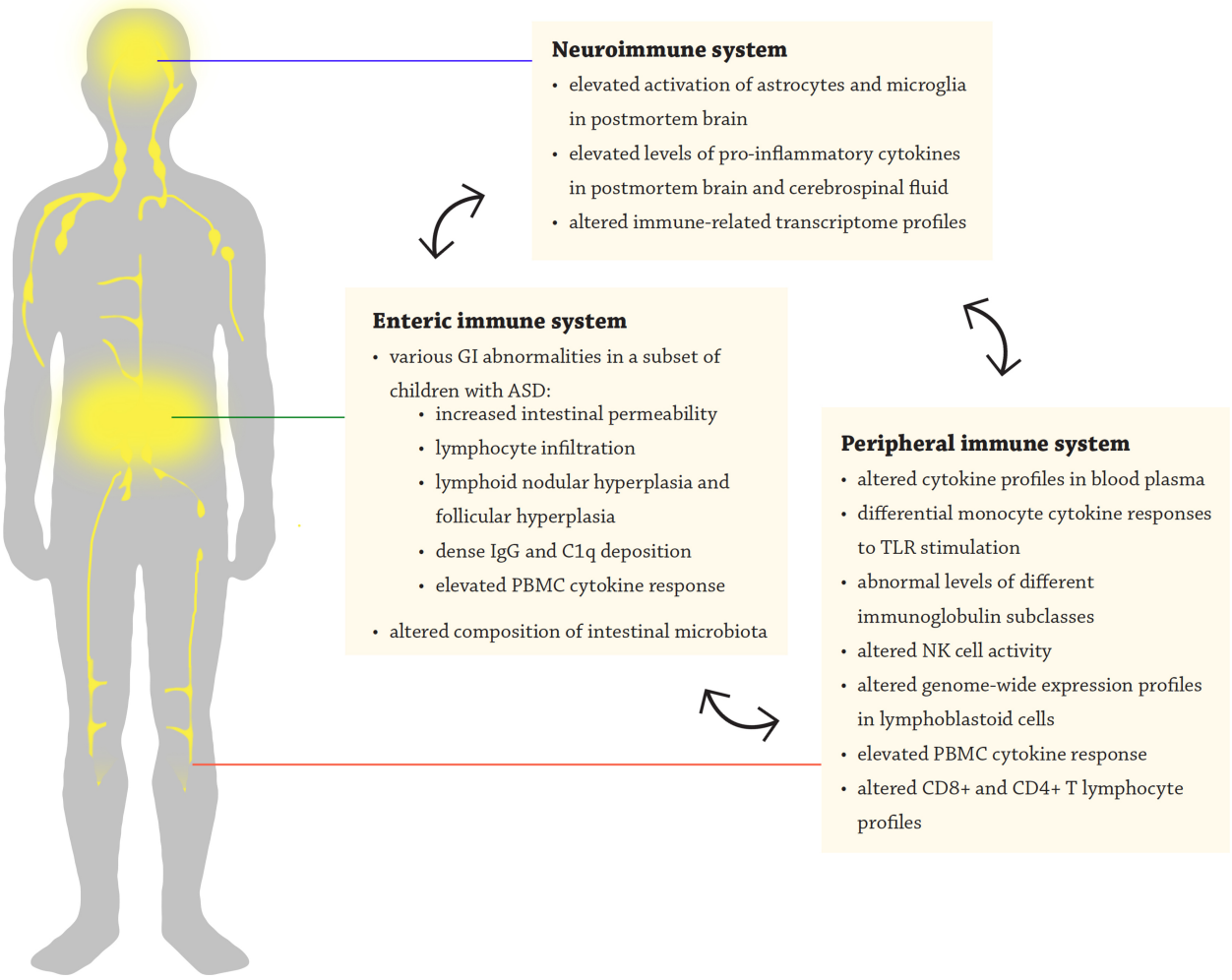

Figure 1. Widespread immune dysregulation may play a role in ASD. Autistic individuals display a variety of immune abnormalities in the brain, periphery and gastrointestinal tract that may contribute to the pathogenesis or maintenance of ASD symptoms.

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Chapter 11

Modeling an autism risk factor leads to permanent immune dysregulation

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Abstract

Increasing evidence highlights a role for the immune system in the pathogenesis of autism spectrum disorders (ASD), as immune dysregulation is observed in the brain, periphery and gastrointestinal tract of ASD individuals. Furthermore, maternal infection (maternal immune activation, MIA) is a risk factor for ASD. Modeling this risk factor in mice yields offspring with the cardinal behavioral and neuropathological symptoms of human ASD. In this study, we find that offspring of immune-activated mothers display altered immune profiles and function, characterized by a systemic deficit in CD4+ TCRβ+ Foxp3+ CD25+ T regulatory cells, increased interleukin-6 (IL-6) and IL-17 production by CD4+ T cells and elevated levels of peripheral **Gr-1+ cells**. In addition, hematopoietic stem cells (HSCs) from MIA offspring exhibit altered myeloid lineage potential and differentiation. Interestingly, repopulating irradiated control mice with bone marrow (BM) derived from MIA offspring does not confer MIA-related immunological deficits, implicating the peripheral environmental context in long-term programming of immune dysfunction. Furthermore, behaviorally-abnormal MIA offspring that have been irradiated and transplanted with immunologically normal BM from either MIA or control offspring no longer exhibit deficits in stereotyped/repetitive and anxiety-like behaviors, suggesting that immune abnormalities in MIA offspring can contribute to ASD-related behaviors. These studies support a link between cellular immune dysregulation and ASD-related behavioral deficits in a mouse model of an autism risk factor.

Introduction

Autism is a complex neurodevelopmental disorder and a pressing medical concern, affecting over 1% of children in the United States (CDC, 2012). While autism spectrum disorders (ASD) are characterized by stereotypic behaviors and language and social deficits, increasing evidence suggests a role for the immune system in ASD pathogenesis. Altered cytokine profiles in postmortem brain, cerebrospinal fluid and plasma are found in ASD, and several studies have demonstrated elevated number and activation of microglia and astrocytes in postmortem brain (Patterson, 2011). There are also many reports of peripheral immune abnormalities in autistic individuals, including increased NK cell activity, differential monocyte responses to *in vitro* stimulation and altered serum immunoglobulin (Ig) levels (P. Goines & Van de Water, 2010; Onore, Careaga, & Ashwood, 2011).

Abnormal activation of the immune system may also be involved in the etiology of autism. Several studies have associated ASD risk with immune-related susceptibility genes, such as MET receptor tyrosine kinase, PRKCB1, complement C4B and specific HLA haplotypes (P. Goines & Van de Water, 2010; Onore et al., 2011). In addition, anti-brain antibodies are elevated in ASD sera and in mothers of autistic children (Croen et al., 2008; Singer et al., 2008; Zimmerman et al., 2007). Family members of autistic children, particularly the mother, show a higher incidence of allergy or autoimmune diseases (Atladottir et al., 2009; Comi, Zimmerman, Frye, Law, & Peeden, 1999). Consistent with immune involvement are findings that maternal infection is a risk factor for autism (Patterson, 2011). After the 1964 rubella pandemic, 8-13% of children born to infected mothers developed features of autism (Chess, 1977). In a recent study surveying

all children born in Denmark from 1980-2005, a very significant association was found between autism and maternal viral infection during the first trimester of pregnancy (Atladottir et al., 2010). Moreover, elevation of IFNγ, IL-4 or IL-5 in maternal serum is associated with increased risk for ASD in the offspring (P. E. Goines et al., 2011), as is elevation of MCP-1 in the amniotic fluid (Abdallah et al., 2011).

Whether the immune abnormalities in ASD actually contribute to its behavioral symptoms or whether they are an epiphenomenon of primary neural dysfunction is an outstanding question. The immune system exhibits lifelong reciprocal interactions with the central nervous system and that immune status can influence behavioral responses is exemplified by early studies demonstrating that responses to infection and inflammation are relayed to the brain, resulting in the induction of fever and sickness behavior (Dantzer, O'Connor, Freund, Johnson, & Kelley, 2008). Moreover, administration of certain cytokines to human subjects frequently causes striking changes in mental state (Capuron & Miller, 2011). Conversely, emotional and psychological state can influence immune function. Perhaps the strongest evidence to date is the finding that both shortand long-term stress lead to disruption of immune function (Segerstrom & Miller, 2004). Immune dysregulation has also been implicated in the etiology of a variety of neurodegenerative, psychiatric and neurodevelopmental disorders, including Parkinson's, Huntington's and Alzheimer's diseases, multiple sclerosis, major depression, schizophrenia and addiction (Amor, Puentes, Baker, & van der Valk, 2010; Dantzer, O'Connor, Lawson, & Kelley, 2011; Patterson, 2007; Shie, Chen, Chen, & Ho, 2011).

Here we ask whether a mouse model exhibiting many features of autism also displays altered immune function. We utilize the maternal immune activation (MIA)

model, which is based on maternal infection as a key environmental risk factor for autism. Pregnant mice are injected with the synthetic, double-stranded RNA, poly(I:C), to initiate a pro-inflammatory, anti-viral response. This type of MIA yields offspring with the core behavioral and neuropathologic**al** symptoms of autism, including reduced social interaction, abnormal communication, stereotyped/repetitive behavior and a spatially-restricted deficit in Purkinje cells (Malkova, Yu, Hsiao, Moore, & Patterson, 2012; L. Shi et al., 2009).

In this study, we profile peripheral immune subtypes, assess the functional activities of major leukocyte lineages and evaluate the lineage potential of fetal and adult hematopoietic stem cells (HSCs) and progenitors. To explore the potential for prenatal programming of long-term immune dysfunction, we examine whether transferring HSCs from MIA offspring into non-MIA offspring can induce cell-autonomous immune abnormalities. To gain insight into whether immune abnormalities in MIA offspring contribute to the pathogenesis of ASD-related behaviors, we behaviorally assess poly(I:C) offspring repopulated with bone marrow from saline offspring. Our findings demonstrate that immune challenge during prenatal life leads to persistent immune alterations in the postnatal offspring, which can further impact the development or maintenance of abnormal behavior.

Results

MIA offspring exhibit deficits in T regulatory cells and elevated CD4+ T cell responses.

Many studies of immune dysregulation in human ASD report a bias toward a pro-inflammatory phenotype (P. Goines & Van de Water, 2010; Onore et al., 2011). We

therefore investigated regulatory T cells (Tregs) as known suppressors of the innate and adaptive immune responses. Compared with controls, adult offspring of poly(I:C)-injected mothers display a ~50% decrease in splenic CD4+ Foxp3+ CD25+ Tregs (Fig. 1A). This deficit is also reflected in significantly decreased levels of total CD4+ Foxp3+ cells and possible decreases ($P = 0.0846$) in CD4+ Foxp3+ CD25− T cells. Similar deficits in total CD4+ Foxp3+, Foxp3+ CD25−, and Foxp3+ CD25+ cells are also observed in mesenteric lymph nodes (MLNs) from adult poly(I:C) offspring (Fig. S1A). Despite these differences in Treg levels, there is no difference in the suppression of CD4+CD25− T-cell proliferation between Tregs from saline versus poly(I:C) offspring (Fig. S2). Taken together, these findings indicate a systemic deficit in the abundance of Tregs.

We further tested MIA offspring for levels of IL-17–producing CD4+ T (Th17) cells, given their proinflammatory nature and reported reciprocal relationship with Tregs. In assays done in parallel with those for Tregs, we find no significant difference from controls in levels of CD4+ TCRβ+ IL-17+ (Th17) cells, with or without IFN-γ expression (Fig. 1B). There is also no difference in the level of CD4+ TCRβ+ IL-17− IFN-γ+ (Th1) cells. To further examine CD4+ T cells, we measured their secretion of IL-6 and IL-17 in response to in vitro stimulation. Compared with controls, CD4+ cells from spleens of 15-wk-old poly(I:C) offspring release significantly more IL-6 and IL-17 after in vitro stimulation, with no difference in TNF-α secretion (Fig. 1C). This result is similarly observed with splenic CD4+ T cells derived from 3-wk-old offspring (Fig. S3A) and 1-y-old offspring (Fig. S3B), suggesting an early onset of persistent immune dysfunction. Interestingly, splenic CD4+ T cells from 3-wkold mice produce much lower levels of IL-

17 than do such cells from adult offspring (0–10 pg/mL compared with 50–400 pg/ mL), reflecting the immunological immaturity reported in young versus adult mice and humans (Adkins, Leclerc, & Marshall-Clarke, 2004). CD4+ T cells from MLNs of poly(I:C) offspring are also hyper-responsive to *in vitro* stimulation, suggesting that this abnormality is common to secondary lymphoid organs (Fig. S1*C*, *D*). Overall, MIA leads to lower Treg levels and elevated CD4+ T cell responsiveness in spleens and MLNs from MIA offspring. This can be characterized as a persistent, pro-inflammatory T helper cell phenotype.

MIA offspring display increased levels of Gr-1+ cells and skewed HSC differentiation

To determine whether MIA during fetal development alters the profile of other immune subtypes in the offspring, we assessed major leukocyte classes in spleens from poly(I:C) and saline offspring. Compared with controls, adult poly(I:C) offspring exhibit a 1.5-fold higher level of Gr-1+ cells and trending increase in CD11b+ cells ($P = 0.1256$) (Fig. 2A). In contrast, there is no difference from controls in the percentages of total B220+ B cells, NK1.1+ NK cells, CD4+ T cells, or CD8+ T cells. Moreover, no significant differences are detected for any of the primary leukocyte subtypes in the MLNs (Fig. S1B).

Gr-1+ cells reflect a heterogeneous group of immune subtypes that includes neutrophils and monocytes, inflammatory cells, and suppressor cells, alike. To determine whether the elevated levels of Gr-1+ cells observed in poly(I:C) spleens could be attributed to particular Gr-1+ subtypes, we further characterized the splenic Gr-1+ population using CD11b, Ly6C, and Ly6G markers. Interestingly, poly(I:C) offspring

exhibit mild increases in all three populations of Gr-1+ cells resolved: (i) Gr-1hi CD11b+ Ly6Cmid Ly6Ghi SSCmid, (ii) Gr-1mid CD11b+ Ly6Cmid Ly6Gmid SSCmid, and (iii) Gr-1mid CD11b+ Ly6Chi Ly6GSSClo (Fig. S4). Gr-1+ subtypes i and ii are referred to as neutrophils, by histology and in line with their high granularity, and subtype iii is identified as a monocyte population (C. Shi et al., 2011). Overall, that poly(I:C) offspring display increases in all identified Gr-1+ populations suggests that skewing at the HSC or progenitor level may underlie the elevated Gr-1 phenotype observed in adult poly(I:C) offspring compared with controls. +

To evaluate the origin of the increase in splenic Gr-1 we assessed the lineage potential and differentiation of BM cells from adult poly(I:C) versus saline offspring. Using a colonyforming assay to morphologically assess lineage differentiation, we find that HSCs and progenitors from poly(I:C) offspring BM exhibit increased differentiation into CFU-G (granulocyte) precursors and decreased differentiation into early CFU-GM (granulocyte-macrophage) precursors (Fig. 2B). Turning to the fetus, we find that similar differentiation is observed with fetal liver HSCs and progenitors from poly(I:C) offspring (Fig. 2C). Thus, MIA induces preferential differentiation of fetal as well as adult HSCs and progenitors into granulocyte precursors, which may account for the increased levels of mature Gr-1+ cells in spleens of poly(I:C) offspring compared with controls.

Immune abnormalities observed in MIA offspring are not transferred via bone marrow transplant.

Our data demonstrate that MIA leads to an altered profile of peripheral immune cells in the offspring, characterized by decreased levels of Tregs, hyperresponsive CD4+

T cells, and elevated levels of Gr-1+ cells. To explore whether these immune abnormalities (and the Gr-1 phenotype, in particular) can be attributed to cell-intrinsic developmental programming of HSCs, we transferred BM from the immunologically aberrant poly(I:C) offspring into irradiated saline and poly(I:C) offspring and assessed whether any MIA-associated immune abnormalities were reestablished.

We find that saline and poly(I:C) offspring that were irradiated and reconstituted with poly(I:C) BM do not develop or retain any of the immune abnormalities exhibited by unmanipulated MIA offspring. All BM-transplanted groups exhibit levels of splenic CD4+ TCRβ+ CD25+ Foxp3+ Tregs and Gr-1+ cells that are comparable to those observed in untransplanted saline offspring (Fig. 3 A and B). There is also no difference between saline and BM-transplanted mice in distributions of progenitor subtypes after in vitro differentiation of BM HSCs (Fig. S5). Furthermore, CD4+ T cells isolated from spleens of any of the types of BM-transplanted mice secrete levels of IL-6 and IL-17 in response to phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation that are similar to those produced by CD4+ T cells isolated from spleens of untransplanted saline offspring (Fig. 3C). Importantly, the fact that saline mice transplanted with saline BM display equivalent levels of Tregs and Gr-1+ cells and similar CD4+ T-cell responsiveness to those observed in unmanipulated saline offspring suggests that the irradiation and BM-transplant procedure itself does not skew immune system profiles under these conditions. Overall, these experiments indicate that MIA-associated immune abnormalities are not transferred via transplant of poly(I:C) BM into irradiated saline or poly(I:C) offspring. This finding suggests either that the HSC microenvironment is important for maintaining altered HSC potential in MIA offspring, or that the particular

MIAassociated immune abnormality is not programmed at the stem cell level.

Bone marrow transplant in MIA offspring normalizes repetitive and anxiety-like behavioral abnormalities

To investigate whether the immune abnormalities found in MIA offspring are an independent parallel pathology or if they actually contribute to the development or maintenance of ASD-like behaviors, we assessed behavioral performance in poly(I:C) offspring transplanted with saline BM. Poly(I:C) offspring were first confirmed to exhibit their expected behavioral phenotypes before the procedure. Compared with controls, poly(I:C) offspring display a deficit in prepulse inhibition (PPI) (Fig. S6A). PPI is used to measure sensorimotor gating of the startle reflex, and decreased PPI is frequently observed in autistic individuals (Perry, Minassian, Lopez, Maron, & Lincoln, 2007). Poly(I:C) offspring also exhibit core behavioral symptoms of autism, including increased repetitive behavior as measured by higher levels of stereotypic marble burying and decreased social preference, as assessed by reduced duration spent and entries into a chamber housing a novel mouse versus a familiar mouse (Fig. S6 B and C). In addition, MIA offspring exhibit increased anxiety, as reflected byeduced duration spent, and entries into, the center arena of an open field, despite no significant difference in total distance traveled (Fig. S6D).

After this initial behavioral testing, the mice were irradiated and transplanted with donor BM harvested from either adult saline or poly(I:C) offspring. Notably, mouse heads were shielded during the procedure to limit known effects of irradiation on neurogenesis and glial activation, and to preclude any associated downstream influences

on behavioral performance. Furthermore, MIA offspring do not exhibit global changes in levels of activated or total brain macrophages/microglia (Fig. S7), suggesting that microglial abnormalities do not contribute the persistence of ASD-related behaviors in these mice.

After irradiation and transplantation with saline BM, MIA offspring no longer exhibit behavioral abnormalities in several of these tests. Irradiation and saline BM transplant in poly(I:C) offspring restores repetitive marble burying to levels observed in saline controls (Fig. 4A). In addition, irradiated and BM-transplanted MIA offspring exhibit significantly decreased anxiety-like behavior, as measured by elevated duration of time spent and entries into the center arena of the open field (Fig. 4B). Interestingly, both saline and poly(I:C) BM-transplanted mice exhibit significantly increased number of center entries compared with untransplanted offspring. This finding is similarly reflected by a statistically significant increase in total distance traveled by transplanted offspring versus untransplanted controls. This finding may be a result of effects of the irradiation and transplant procedure on behavior rather than habituation of the mice to repeated testing, because we are able to replicate open field deficits of equivalent intensity in poly(I:C) offspring after retesting at different adult ages (Fig. S8). On the other hand, treatment of poly(I:C) offspring with saline BM has no significant effect on the deficit in social preference, as measured by duration and number of entries into the social chamber (Fig. 4C).

Although treatment of MIA offspring with irradiation and saline BM transplantation significantly restores normal behavior in several tests, we find that treatment of MIA offspring with irradiation and poly(I:C) BM transplantation also

improves those behaviors. Notably, irradiated MIA offspring transplanted with poly(I:C) BM exhibit improved open field and marbleburying performance (Fig. S9). This result is consistent with our finding that transplant of poly(I:C) BM does not give rise to cellautonomous immune abnormalities (Fig. 3). As such, both poly (I:C) BM and saline BM can be considered immunologically normal in these experiments. Taken together, these data demonstrate that MIA offspring that had previously exhibited abnormal behaviors fail to exhibit repetitive and anxiety-like behavior after exposure to irradiation and BM transplant.

Discussion

In the present study, we identify differences in dynamic cellular immune responses in a mouse model of an autism risk factor. Offspring of immune-activated mothers develop altered immune profiles and function in the spleen and MLN, which are consistent with a pro-inflammatory phenotype. In addition, we demonstrate preferential myeloid differentiation of fetal HSCs and progenitors, which is also found in adult HSCs. This could form the basis for the altered immune distributions in secondary lymphoid organs from MIA offspring. However, BM transplant of HSCs derived from MIA offspring is not sufficient to recapitulate the elevated levels of Gr-1+ cells exhibited by untransplanted MIA offspring. This result highlights the importance of appropriate environmental cues for preserving this phenotype, as discussed below. We also used the irradiation-BM transplant approach to explore the role of peripheral immune dysfunction on behavioral performance. Interestingly, this procedure corrects some of the ASD-like behavioral symptoms in the MIA offspring.

MIA leads to permanently hyper-responsive CD4+ T cells as well as decreased Tregs in the offspring, suggesting a chronic, pro-inflammatory phenotype. This finding is consistent with decreased numbers CD4+ CD25+ and CD3+GITR+ T cells observed in children with ASD (Ashwood et al., 2011b; Mostafa, Al Shehab, & Fouad, 2010). Diminished Treg levels and associated decreases in immune regulation may reflect the finding that autistic individuals exhibit decreased levels of regulatory cytokines, such as TGFβ1, and increased levels of pro-inflammatory cytokines in serum, cerebrospinal fluid and postmortem brain (Patterson, 2011). Furthermore, given that Tregs are critical for limiting immune activation and preventing self-reactivity, their deficiency may underlie the reports of a link between ASD and autoimmune disease (Atladottir et al., 2009; Enstrom, Van de Water, & Ashwood, 2009).

Our finding that CD4+ T cells from MIA offspring are hyper-responsive to *in vitro* stimulation further reflects diminished immune homeostasis. Increases in activated DR+ T cells are observed in human autism (Plioplys, Greaves, Kazemi, & Silverman, 1994; Warren, Yonk, Burger, Odell, & Warren, 1995). In addition, elevated levels of TNFαand IFNγ-producing T cells are found in peripheral blood and gastrointestinal mucosa from autistic individuals (Ashwood & Wakefield, 2006). A number of other altered immune responses have been reported in ASD (P. Goines & Van de Water, 2010; Onore et al., 2011), and we find that MIA offspring also exhibit some of these changes, including altered leukocyte subsets and CD4+ T cell responses to stimulation. This overlap between findings of immune dysregulation in ASD and our current results lends support to MIA as a mouse model with construct and face validity for this disorder.

We further demonstrate that MIA during fetal development leads to significantly increased levels of peripheral Gr-1+ CD11b+ neutrophilic and monocytic cells in adult offspring. Granulocytosis is typically observed after acute inflammation (Hansen, Karle, Andersen, Malmquist, & Hoff, 1976; Ricevuti, Mazzone, Pasotti, de Servi, & Specchia, 1991) and is also a feature of a number of chronic diseases (Cascao, Rosario, Souto-Carneiro, & Fonseca, 2010; Courtney et al., 1999; Passegue, Jochum, Schorpp-Kistner, Mohle-Steinlein, & Wagner, 2001). There have been no reports of altered neutrophil levels in ASD, however. In one study, children with ASD exhibited increased plasma monocyte counts, but no significant abnormalities in major granulocyte subtypes (Sweeten, Posey, & McDougle, 2003). In light of the present results, it will be of interest to extend these immunophenotypic studies to behavioral or symptomatic subpopulations of ASD individuals.

It is intriguing to consider that MIA effects on developing HSCs may underlie some of the persistent peripheral immune changes found in the offspring. We find that both adult and fetal HSCs and progenitor cells display preferential differentiation into CFU-G colonies, which provides an explanation for how increases in such short-lived cells are maintained over the lifespan of MIA offspring. Maternal poly(I:C) injection is known to induce dramatic increases in pro-inflammatory cytokines in the placenta, a principal HSC niche during midgestation (Hsiao & Patterson, 2011). Since HSCs and progenitors respond to inflammatory signals such as cytokines and TLR ligands, it will be interesting to explore whether MIA-induced changes in the HSC environment can skew hematopoietic lineage decisions and fate.

Despite the fact that HSCs and progenitors isolated from poly(I:C) offspring are developmentally skewed toward granulocyte precursors, transplantation of BM from poly(I:C) offspring into irradiated saline offspring or back into irradiated poly(I:C) offspring does not transfer the Gr-1 phenotype. This suggests that developmental programming of HSCs to skew lineage differentiation in MIA offspring is not governed solely by stably-encoded cell-intrinsic factors, such as epigenetic modification. Rather, it is likely that an MIA-induced peripheral environment is necessary to supply the cues that guide preferential myeloid development. Changes in G-CSF, IL-5 and IL-3 levels, for example, are implicated in fine-tuning the transcription factor activity that governs lineage choice for granulocyte/macrophage progenitors (Robb, 2007). Indeed, we have found that MIA offspring exhibit dynamic and chronically altered peripheral blood and splenic cytokine profiles (Hsiao, Garay, Patterson, & McAllister, 2011). It will be of interest to assess whether the BM microenvironment differs in poly(I:C) and saline offspring. Overall, it is intriguing that the Gr-1 phenotype observed in MIA offspring is not transferred via BM transplant, suggesting a lack of sufficient cell-intrinsic epigenetic programming and/or a key role for environmental factors in promoting this lineage choice.

We demonstrate that subjecting MIA offspring that have validated behavioral deficits to irradiation and reconstitution with immunologically normal BM alters their behavioral phenotype. Namely, these MIA offspring no longer exhibit their previous abnormalities in repetitive marble burying and open field exploration. These findings imply that correcting immune function can correct some autism-related behavioral abnormalities. However, this experiment has a significant limitation. In the absence of a

positive control (MIA offspring that retain behavioral deficits after irradiation and BM transplantation), we are unable to distinguish between the potential restorative effects of the BM itself (that is, the effects of restoring immune phenotype) versus the effect of confounding factors associated with the transplant procedure. The most obvious of these is the effect of irradiation on various aspects of recipient homeostasis, including metabolic function, GI microbial composition and oxidative stress, all of which may indirectly influence behavioral outcome. We are, however, inspired by recent work demonstrating that stereotyped grooming behavior in *hoxb8* mutant mice is, in fact, transferrable via BM transplant (Chen et al., 2010). This shows, at least in that experimental model, that mice can reconstitute an expected behavioral impairment via BM transplant despite prior irradiation. It will be important to explore other experimental approaches to treat immune dysfunction in MIA offspring and to further assess whether these can alleviate behavioral outcomes.

Nonetheless, our finding that behavioral abnormalities in the MIA offspring can be corrected by the irradiation-BM transplant procedure contributes to a growing number of studies reporting the efficacy of BM transplant in ameliorating symptoms of neurological and psychiatric disorders (Chen et al., 2010; Derecki et al., 2012; Kwan et al., 2012). Furthermore, several experiments utilizing RAG1 KO, SCID and athymic mice demonstrate that primary immune dysfunction can lead to behavioral impairment (Brynskikh, Warren, Zhu, & Kipnis, 2008; Cushman, Lo, Huang, Wasserfall, & Petitto, 2003; Kipnis, Cohen, Cardon, Ziv, & Schwartz, 2004; Mombaerts et al., 1994). Interestingly, work with germ-free mice links the absence of microbiota and associated alterations in the immune system to abnormal behavioral performance (Heijtz et al.,

2011). Some immune alterations have been described in the maternal valproic acid model and the BTBR mouse strain that display behavioral features of autism (Heo, Zhang, Gao, Miller, & Lawrence, 2011; Schneider et al., 2008). In contrast, much remains to be learned about potential immune changes in mouse models of ASD candidate genes. One such gene is particularly attractive in this regard*MET*, which encodes a tyrosine kinase receptor and is known to play a role in immune regulation (Campbell, Li, Sutcliffe, Persico, & Levitt, 2008).

Altered peripheral immune profiles and cellular activity are also associated with core behavioral impairments in human ASD. Increased plasma IL-4 levels correlate with greater deficits in communication, while increased plasma IL-8, IL-12p40, IL-6 and IL-1β are linked to stereotypy, hyperactivity and lethargy scores from ASD children (Ashwood et al., 2011a). Altered levels of other immune factors, including TGF, MIF and CD31, have also been associated with the severity of ASD-related behaviors or pathophysiology (Onore et al., 2011). In addition, reduced levels of plasma IgG and IgM are associated with behavioral severity in ASD children (Heuer et al., 2008).

It is striking that in a mouse model of an autism environmental risk factor that exhibits the cardinal behavioral and neuropathological symptoms of autism, there is also permanent peripheral immune dysregulation. This provides the opportunity to explore molecular mechanisms underlying the relationship between brain dysfunction and altered immunity in the manifestation of abnormal behavior. Furthermore, it provides a platform for investigating how prenatal challenges can program long-term postnatal immunity, health and disease. Maternal insult-mediated epigenetic modification in HSC and progenitor cells is one possible mechanism for how effects may be established by

transient environmental changes yet persist permanently into adulthood. However, the BM transplant results suggest that the peripheral environment of the MIA offspring is also critical for maintaining a permanently modified immune state.

Methods

MIA

Pregnant C57BL/6J mice were injected on E12.5 with saline or poly(I:C). For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO) was dissolved in saline at 4 mg/ml and administered i.p. at 20 mg/kg (based on the weight of the poly(I:C) itself, not including the total weight of the potassium salt). Control mice were injected with saline alone at 5 μl/g body weight.

CD4+ T cell in vitro *stimulation*

10^6 CD4+ T cells were cultured in complete RPMI with PMA (50 ng/mL) and ionomycin (750 ng/mL) for 3 days at 37°C with 5% CO_2. Each day, 0.5 ml supernatant was collected. ELISA assays to detect IL-6, IL-17 and TNFα were performed according to the manufacturer's instructions (eBioscience).

Flow cytometry

For subtyping of Gr-1+ splenocytes, cells were stained with Gr-1-APC, CD11b-PE, Ly6G-APC, Ly6C-FITC and Ter119-PerCP-Cy5.5 (Biolegend; San Diego, CA). For detection of Th17 cells and Tregs, splenocytes were stimulated for 4 hours with PMA/ionomycin in the presence of GolgiPLUG (BD Biosciences). Suspensions were

blocked for Fc receptors and labeled with CD4-FITC, TCRb-PerCP-Cy5.5 and CD25-PE before labeling with IFNγ-PE and Foxp3-APC (eBioscience). Samples were processed using the FACSCalibur cytometer (BD Biosciences). Data were analyzed using FlowJo software (TreeStar).

Treg suppression assay

10^5 CD4+CD25T responder cells were cultured in complete RPMI with 2 x 10^4 irradiated antigen-presenting cells and 2.5 μg/ml anti-CD3, with CD4+CD25+ Tregs at a ratio to T responder cells of 1:1, 1:2 and 1:4, according to methods from (Collison & Vignali, 2011). After 3 days of culture, suspensions were stained with CD4-APC antibody and assessed by flow cytometry.

Methylcellulose colony forming assay

2 x 10^5 fetal liver or BM cells were added to 3 ml of thawed methylcellulose supplemented with SCF, IL-3, IL-6 and Epo (StemCell). Total colony number was counted on day 5 and confirmed to be equivalent across groups. CFU-G, -M, -E, -GM and -GEMM colonies were blindly scored on day 12 according to standard protocols.

BM transplantation

Behaviorally tested, 9-week-old mice were injected i.p. with high dose ketamine/xylazine (5.6 μl/1 g mouse) and lethally irradiated (1000 rads) with heads shielded by 4mm lead. The effectiveness of the head shields was confirmed by selective graying of black coat color in the irradiated area. Recovered mice were anesthetized with isofluorane and

injected retro-orbitally with 5 x 10^6 donor BM cells. Mice were re-tested in behavioral paradigms at 17-19 weeks of age and sacrificed for immunological assays at 19-20 weeks of age.

Behavioral testing

At 6-8 weeks of age, mice were behaviorally tested for pre-pulse inhibition (PPI), open field exploration, repetitive marble burying and social preference (Hsiao & Patterson, 2011; Malkova et al., 2012; Smith, Li, Garbett, Mirnics, & Patterson, 2007; Walf & Frye, 2007). 8 weeks after transplant, mice were similarly tested in all paradigms but PPI since PPI performance is highly sensitive to handling and prior testing experience (Plappert, Kuhn, Schnitzler, & Pilz, 2006).

Statistical analysis

Statistical analysis was performed with Prism software (Graphpad). Differences between two treatment groups were assessed using Student's t-test, and differences among multiple groups were assessed using one-way ANOVA + Bonferroni post-hoc test. Two-way ANOVA + Bonferroni post-hoc test was used for PPI, CD4+ T cell stimulation and methylcellulose assay data.

Mice

C57BL/6J mice (Charles River) were housed under specific pathogen-free conditions in the California Institute of Technology's Broad animal facility. Mice were mated overnight and the presence of a vaginal plug on the following morning was noted as

embryonic day (E) 0.5. All experiments were performed under the approval of the California Institute of Technology Institutional Animal Care and Use Committee.

Spleen and Mesenteric Lymph Node Suspensions

Spleens and mesenteric lymph nodes (MLNs) were harvested, washed in complete RPMI media on ice, and forced through 40-μm cell strainers (BD Biosciences). In some trials, spleens from the same treatment group were pooled in groups of three to facilitate accommodating a large number of samples, while preserving cell viability. Singlecell suspensions were subjected to RBC lysis (Sigma Aldrich). CD4+ T cells were isolated by negative selection using a CD4+ Isolation kit (Miltenyi Biotec). Cell counts were performed on a hemocytometer, and 106 cells were aliquoted for each CD4+ stimulation reaction.

Flow Cytometry

For extracellular staining, cells were treated with anti-mouse CD16/CD32 Fc block (eBioscience) before staining with subsets of these antibody conjugates: Ter119-PerCP-Cy5.5, CD4-FITC, CD8-FITC, Gr-1-APC, B220-FITC, CD11b-APC (eBioscience), NK1.1-PE (Biolegend).

Regulatory T-Cell Suppression Assay

For regulatory T-cell (Treg) suspression assay, 105 CD4+CD25− T-responder cells were cultured in complete RPMI with 2 × 104 irradiated antigen-presenting cells and 2.5 μg/mL anti-CD3, with CD4+CD25+ Tregs at a ratio to T-responder cells of 1:1, 1:2, and

1:4, according to methods from (1). After 2 d of culture, suspensions were stained with CD4-APC antibody and assessed by flow cytometry.

Fetal Liver Suspensions

Fetal livers were harvested from E13.5 and E15.5 embryos and washed in PBS. Six to seven fetal livers from a single litter were pooled, forced through a 40-μm cell strainer, and washed with Iscove's modified Dulbecco's medium (IMDM) + 2% (vol/vol) FBS (StemCell Technologies). Cells were counted using a hemocytometer, and 2 × 105 cells used per methylcellulose solution.

Donor Cell Harvest for Bone Marrow Transplantation

Four adult saline offspring (two male, two female) and four adult poly(I:C) offspring (two male, two female) were selected from an independent cohort of behaviorally tested maternal immune activation (MIA) and control mice, and designated as bone marrow (BM) donors. Donor adult saline and poly(I:C) mice were killed by CO2 gas, and BM was harvested by flushing femurs and tibias with DMEM (Thermo Scientific). BM suspensions from each treatment group were pooled, subjected to RBC lysis (Sigma Aldrich), filtered through a 40-μm cell strainer, and stored on ice.

Behavioral Testing

For prepulse inhibition (PPI), mice were acclimated to an SR-LAB testing chamber (SD Instruments) for 5 min, presented with six, 120-dB pulses of white noise (startle stimulus), and then subjected to 14 randomized blocks of either no startle, startle stimulus

only, 5-dB prepulse + startle, or 15-dB prepulse + startle. The startle response was recorded by a piezoelectric sensor, and PPI defined as: (startle stimulus only – 5 or 15 dB prepulse + startle)/startle stimulus only × 100.

For open-field testing, mice were allowed to explore a 50 × 50-cm white Plexiglas box for 10 min. An overhead video camera was used to record the session, and Ethovision software (Noldus Information Technology) used to analyze the distance traveled, and the number of entries and duration of time spent in the center arena (central square, 17 × 17 cm).

For marble-burying testing, mice were acclimated for 10 min to a new testing cage containing Aspen pine bedding (NEPCO), 3-cm deep. The mice were briefly placed in a fresh holding cage while 18 navy blue marbles (15-mm diameter, washed with 70% ethanol and mixed in Aspen pine bedding) were laid in a 6 × 3 pattern in the testing cage. Mice were placed back to the testing cage, and the number of buried marbles (>50% covered with bedding) was scored after a 10-min trial.

For social interaction testing, mice were first habituated for 10 min to a 60 × 40-cm Plexiglas box with three equally-sized chambers, with each of the two side chambers containing an empty Plexiglas cylinder. During a second 10-min trial, the mouse was placed in the center chamber and given the choice to interact with an unfamiliar, same-sex mouse that was placed in one of the side chamber cylinders, or with a nonsocial object (novel green, sticky ball toy), placed in the other side chamber cylinder. In the third and final 10-min trial, social preference was measured by replacing the nonsocial object with a new, unfamiliar, same-sex mouse. In this trial, the mouse was given the choice to interact with the now familiar mouse (from trial 2) or the new unfamiliar

mouse. The cylinders holding the stimulus mice or the toy have small holes (~10 mm diameter) over the entire surface area to permit direct interaction. Mice to be tested were prehabituated to the Plexiglas cylinders and three-chambered box for 20 min each day for 3 d before testing. All equipment was cleaned with 70% ethanol and Process NPD (STERIS Life Sciences) before and after testing. The side chamber used for placement of the social object or the nonsocial object, and the familiar mouse or the unfamiliar mouse was alternated in each trial to prevent bias. The duration of time spent and number of entries into each chamber were recorded using an overhead video camera and analyzed with Ethovision software. Equivalent levels of chamber duration and chamber entries during the habituation phase were used to ensure that the mice did not exhibit chamber bias. “Social preference, chamber duration” is measured by: (duration in the novel mouse chamber – duration in the familiar mouse chamber). “Social preference, chamber frequency” is measured by: number of entries into the novel mouse chamber – number of entries into the familiar mouse chamber. For post-BM transplant behavior testing, different stimulus (familiar and unfamiliar) mice were used to prevent potential confounding effects of social memory.

Footnotes

EYH, PHP & SKM designed research; EYH, SM & JC performed research; EYH & JC analyzed data; EYH, SM, JC, PHP & SKM wrote the paper.

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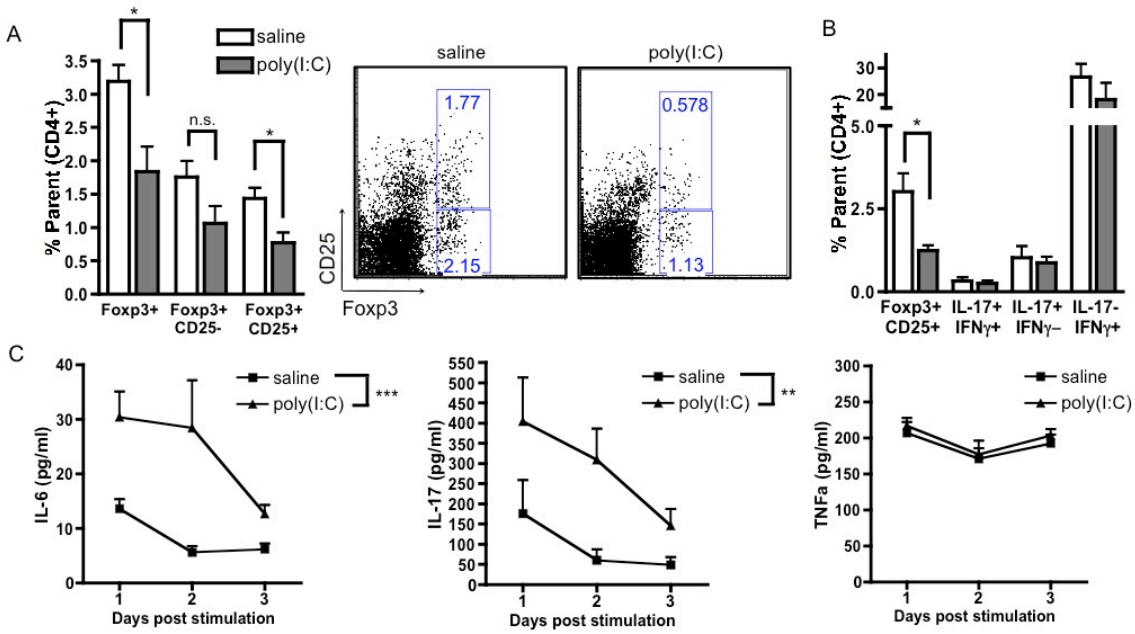

Fig. 1. MIA leads to decreased levels of Tregs and hyperresponsiveness in CD4+ T cells from spleens of adult offspring. (A) Compared with controls, adult poly(I:C) offspring exhibit decreased levels of CD4+ Foxp3+ splenocytes and CD4+ Foxp3+ CD25+ Tregs (n = 5, where each sample represents a pool of three spleens). (B) Adult poly(I:C) offspring exhibit decreased levels of splenic CD4+ TCRβ+ Foxp3+ CD25+ Tregs, but no significant difference in splenic CD4+ TCRβ+ IFN-γ+ IL-17+ Th17+ Th1 cells or CD4+ TCRβ+ IFN-γ− IL-17+ Th17 cells (n = 4, where each sample represents a pool of three spleens). (C) CD4+ T cells from the spleens of adult offspring secrete elevated levels of IL-6 and IL-17 in response to PMA/ionomycin stimulation. In contrast, CD4+ T cells from spleens of adult poly(I:C) and saline offspring do not differ the level of TNF-α secreted (n = 11–16). *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant. All panels show one representative experiment of at least two separate trials.

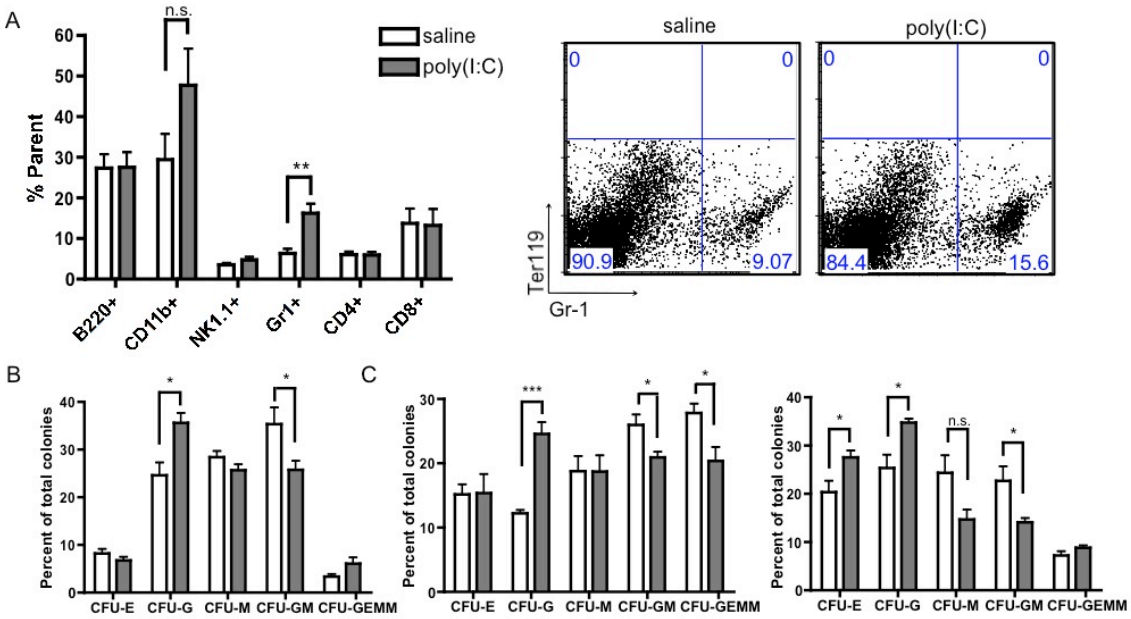

Fig. 2. MIA leads to increased levels of splenic Gr-1+ cells in adult offspring and preferential differentiation of HSCs into granulocyte precursors in fetal and adult offspring. (A) Spleens from adult poly(I:C) offspring exhibit increased levels of Gr-1+ cells and no significant differences in other major lineages compared with controls (n = 4, where each sample represents a pool of three spleens). (B) Compared with BM HSCs from control offspring, BM HSCs from adult poly(I:C) offspring display increased differentiation into CFU-G precursors and decreased differentiation into early CFU-GM precursors (n = 4). Compared with controls, fetal liver HSCs from embryonic day (E) 13.5 (C, Left) and E15.5 (C, Right) poly(I:C) offspring also display increased differentiation into CFU-G and decreased differentiation into CFU-GM, in addition to decreased percentages of CFU-GEMM with E13.5 HSCs and increased percentages of CFU-E with E15.5 HSCs (n = 4, where each sample represents a pool of cells from six fetal livers from a single litter). *P < 0.05, **P < 0.01, ***P < 0.001; n.s., not significant. All panels represent one representative experiment of at least two separate trials.

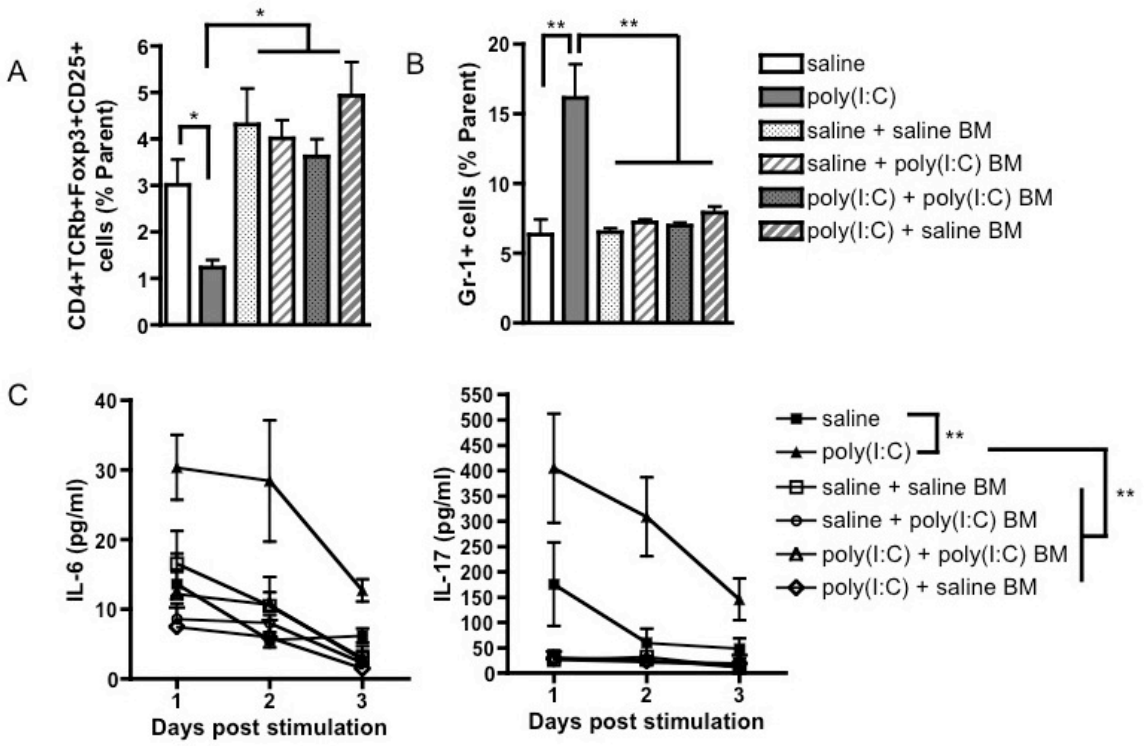

Fig. 3. Immune abnormalities observed in MIA offspring are not transferred by BM transplant into irradiated mice. (A) There is no difference in levels of splenic CD4+ TCRβ+ CD25+ Foxp3+ Tregs between saline or poly(I:C) offspring transplanted with BM from saline or poly(I:C) offspring. Levels of splenic Tregs between BM transplant groups are comparable to those observed in untransplanted saline offspring; untransplanted poly(I:C) offspring display a significant deficit in Treg percentages (n = 4–5). (B) BMtransplanted mice do not display MIA-associated increases in splenic Gr-1+ cells. Percentages of Gr-1+ cells are comparable between BM transplanted groups and untransplanted saline offspring (n = 4–5), but untransplanted poly (I:C) offspring exhibit significantly elevated levels of splenic Gr-1+ cells. (C) CD4+ T cells from BM-transplanted mice exhibit statistically equivalent levels of IL-6 (Left) and IL-17 (Right) in response to PMA/ionomycin stimulation in vitro. There are no differences between

concentrations of IL-6 and IL-17 secreted by CD4+ cells from BM-transplanted groups and from untransplanted saline offspring, despite significant hyperresponsiveness of CD4+ T cells from poly(I: C) offspring [n = 11–16 for saline and poly(I:C) groups, 4–5 for BM transplant groups]. *$P < 0.05$, **$P < 0.01$. BM transplant data were acquired from one large experiment.

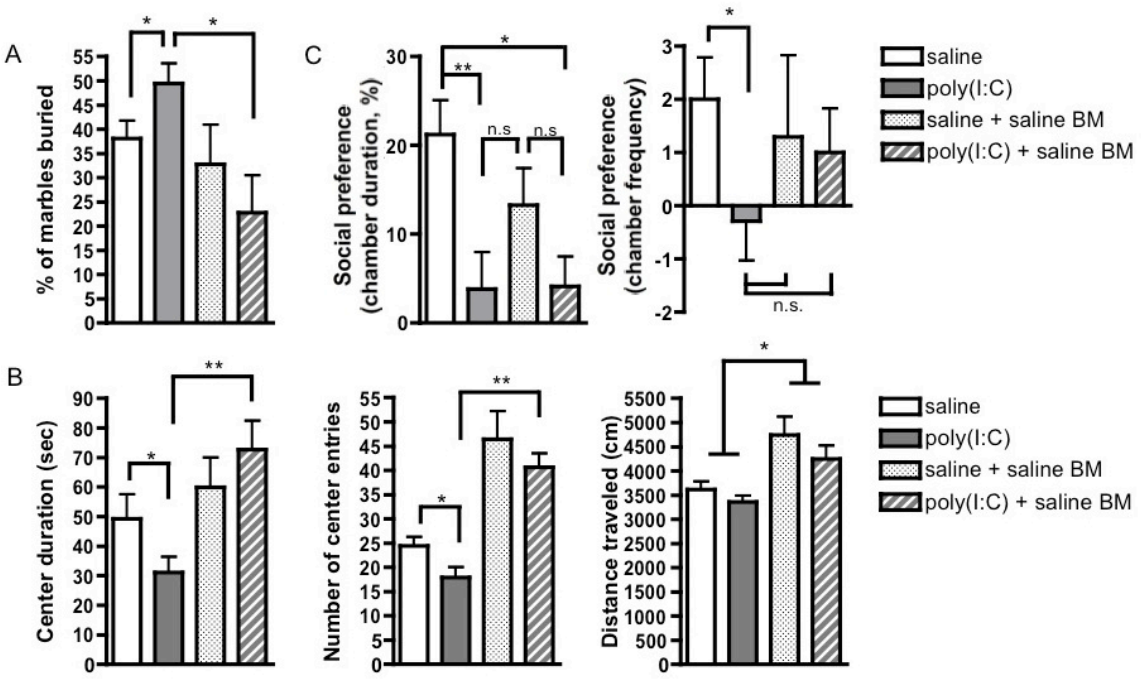

Fig. 4. MIA offspring irradiated and transplanted with saline BM exhibit decreased repetitive and anxiety-like behavior. (A) Irradiation and transplantation of saline BM into MIA offspring restores repetitive marble-burying behavior to a level at or below that observed in controls. (B) Irradiation and transplantation of saline BM into MIA offspring decreases anxiety-like behavior as measured by significant increases in the number of center entries (Center) and duration in the center arena (Left), compared with those observed in untransplanted MIA mice. Both saline and poly(I:C) BM-transplant mice also exhibit increased overall activity in the open field, as measured by significantly increased total distance traveled compared with untransplanted mice (Right). (C) Transplanting saline offspring BM into poly(I:C) offspring has no significant effect on their social preference behavior, as measured by chamber duration (Left). There is, however, a trending improvement in social preference behavior as measured by chamber frequency in BM-transplanted MIA offspring (Right). *$P < 0.05$, **$P < 0.01$; n.s., not significant. BMtransplant data were acquired from one large experiment.

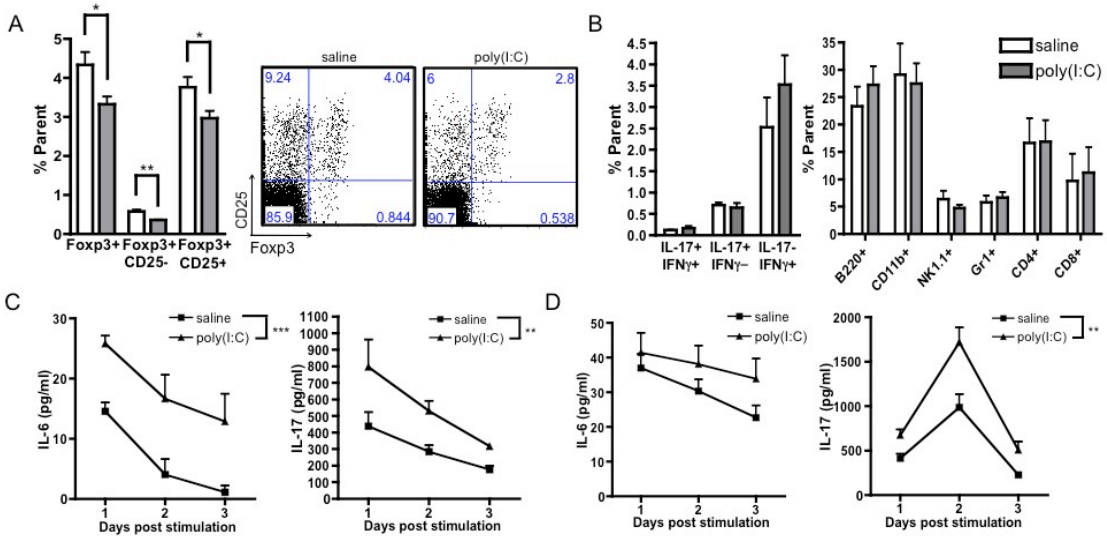

Fig. S1. Some of the immune changes observed in spleens of MIA offspring are recapitulated in the MLN. (A) Consistent with what is observed in the spleen, MLNs from poly(I:C) offspring exhibit decreased levels of total CD4+ Foxp3+, CD4+ Foxp3+ CD25−, and CD4+ Foxp3+ CD25+ T cells, but no significant difference in levels of CD4+ TCRβ+ IL-17+ IFN-γ+ Th17+ Th1 cells or CD4+ TCRβ+ IL-17+ IFN-γ− Th17 cells (n = 5, where each sample represents a pool of three spleens). (B) There are no significant differences between MLNs from adult poly(I:C) versus saline offspring in the distribution of major leukocyte classes [n = 8 saline, 9 poly(I:C)]. (C) Consistent with what is observed in the spleen, CD4+ T cells from the MLNs from 15-wk-old poly(I:C) offspring produce increased levels of IL-6 and IL-17 at 1, 2, and 3 d after phorbol 12-myristate 13-acetate (PMA)/ionomycin stimulation (n = 4, where each represents a pool MLNs from three animals). (D) This hyperresponsive phenotype of MLN CD4+ T cells is maintained in 1-y-old poly(I:C) versus saline mice (n = 11–16). *P < 0.05, **P < 0.01, ***P < 0.001. All panels represent one representative experiment of at least two separate trials.

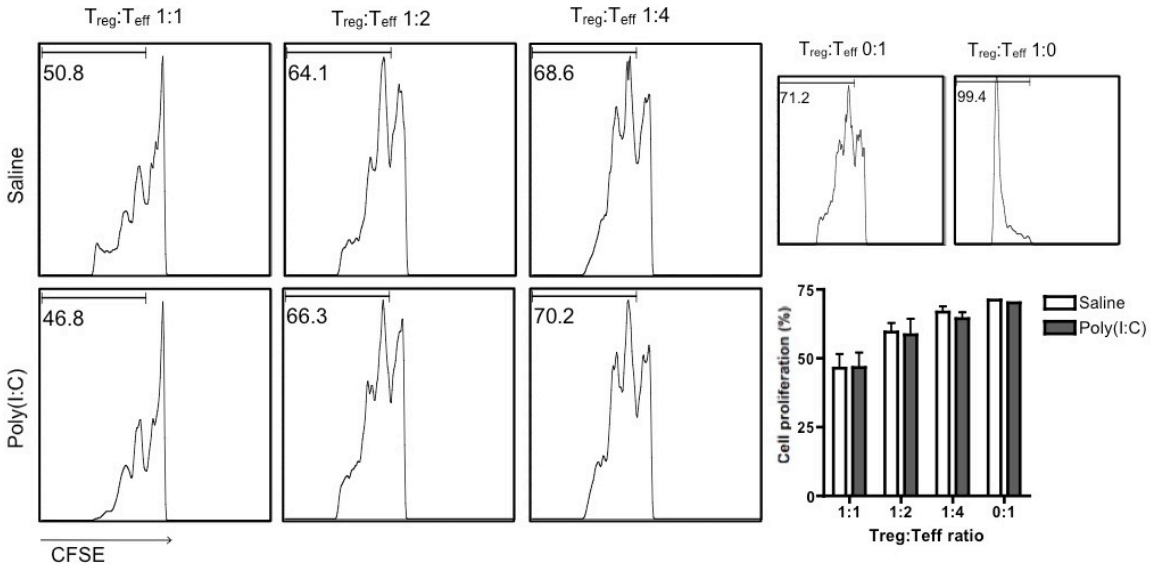

Fig. S2. There is no significant difference in the suppression of CD4+CD25− T-cell (effector T cells, Teff) proliferation by splenic Tregs from adult saline versus poly(I:C) offspring. Similar levels of Teff proliferation are observed after 48 h of coculture of Tregs with Teffs at ratios of 1:1, 1:2, and 1:4 (n = 4).

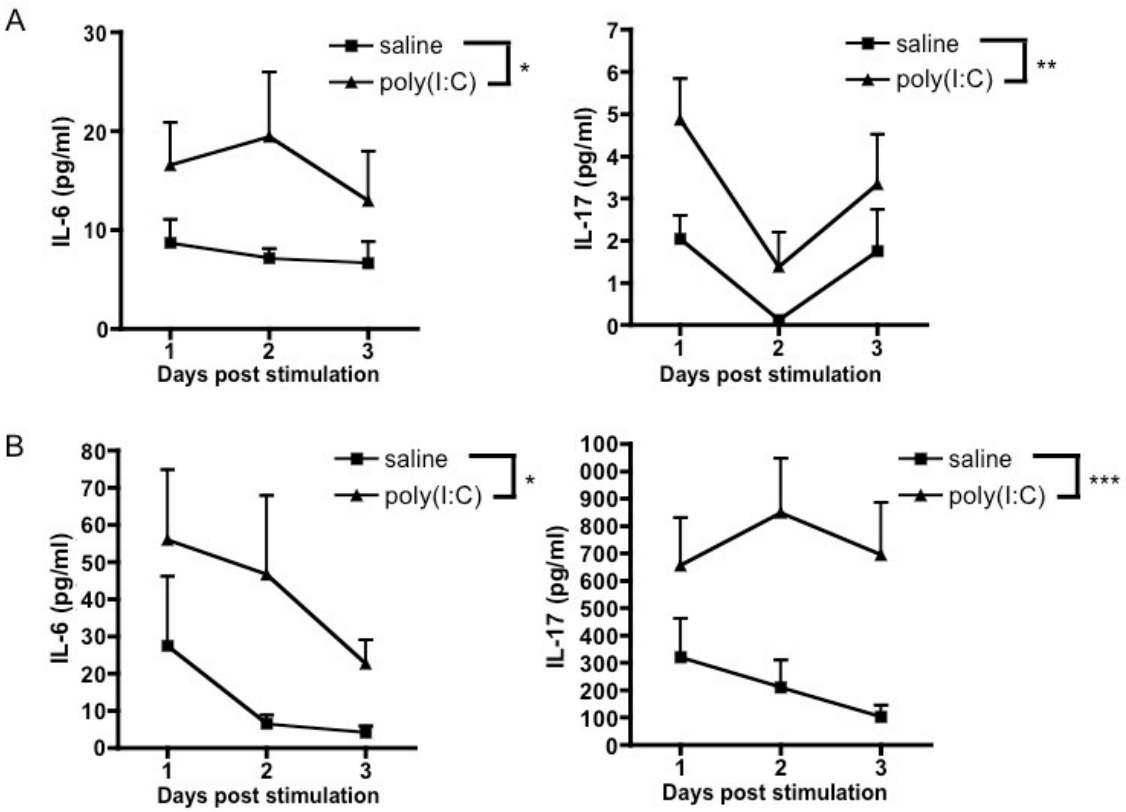

Fig. S3. Hyperresponsiveness of CD4+ T cells from MIA offspring is established early in development and maintained through adulthood. CD4+ T cells from the spleens of 3-wk-old MIA offspring (A) and 1-y-old MIA offspring (B) secrete elevated levels of IL-6 (Left) and IL-17 (Right) in response to PMA/ionomycin stimulation (n = 5–7). *P < 0.05, **P < 0.01, ***P < 0.001. All panels represent one representative experiment of at least two separate trials.

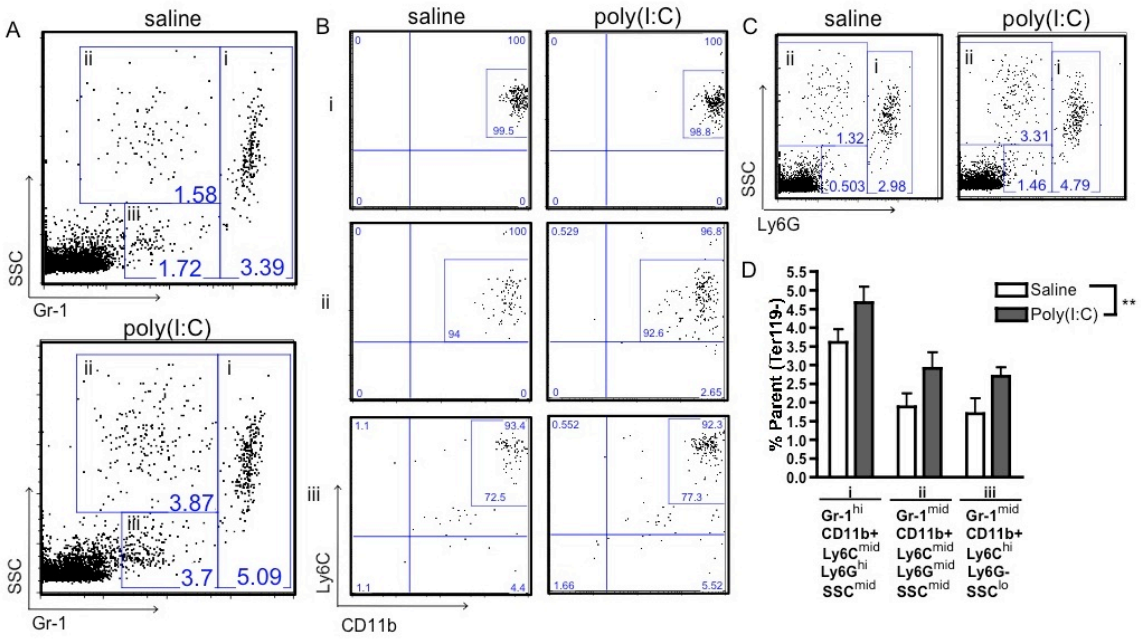

Fig. S4. Increases in both neutrophilic and monocytic Gr-1+ CD11b+ cells constitute the MIA-associated increase in total Gr-1+ cells. (A) Three populations of Gr-1+ cells are resolved based on Gr-1 intensity and granularity (side scatter, SSC). (B) Further stratification by CD11b and Ly6C intensity demonstrates that all three populations are CD11b+ and Ly6C+ (high or mid). (C) Assessment for Ly6G intensity reveals monocytic Gr-1+ cells [Ly6G−, population (iii)] and neutrophilic Gr-1+ cells [Ly6G high or mid, populations (i) and (ii)]. (D) Adult poly(I:C) offspring exhibit mild increases in all three populations of Gr-1+ cells identified: (i) Gr1hi CD11b+ Ly6Cmid Ly6Ghi SSCmid, (ii) Gr-1mid CD11b+ Ly6Cmid Ly6Gmid SSCmid, and (iii) Gr-1mid CD11b+ Ly6Chi Ly6G− SSClo, which result in a statistically significant difference based on treatment group (n = 5). **P < 0.01.

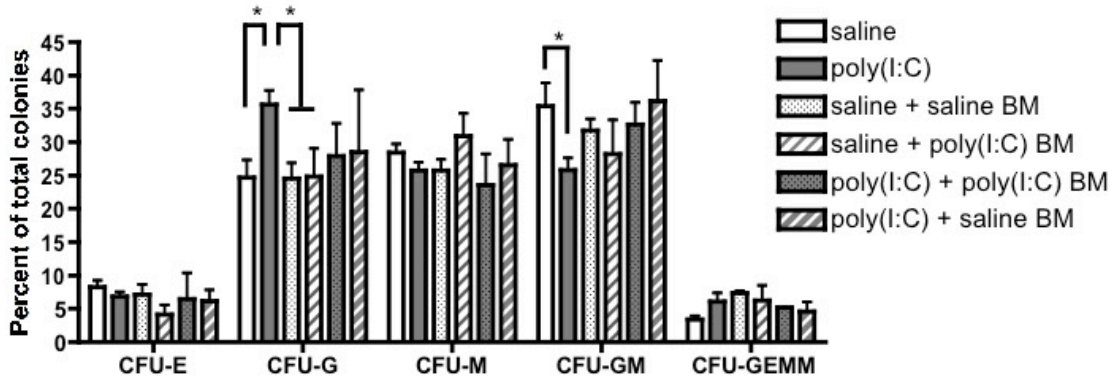

Fig. S5. There is no significant difference between unmanipulated saline mice and BM-transplanted mice in the lineage potential of BM HSCs and progenitors differentiated in vitro. Transplant of poly(I:C) or saline BM into irradiated poly(I:C) offspring corrects the abnormal lineage differentiation phenotype associated with unmanipulated poly(I:C) offspring. Similarly, transplant of poly(I:C) BM into irradiated saline offspring does not transfer the preferential skewing toward CFU-G (granulocyte) colonies that is observed in unmanipulated poly(I:C) offspring (n = 3). *$P < 0.05$.

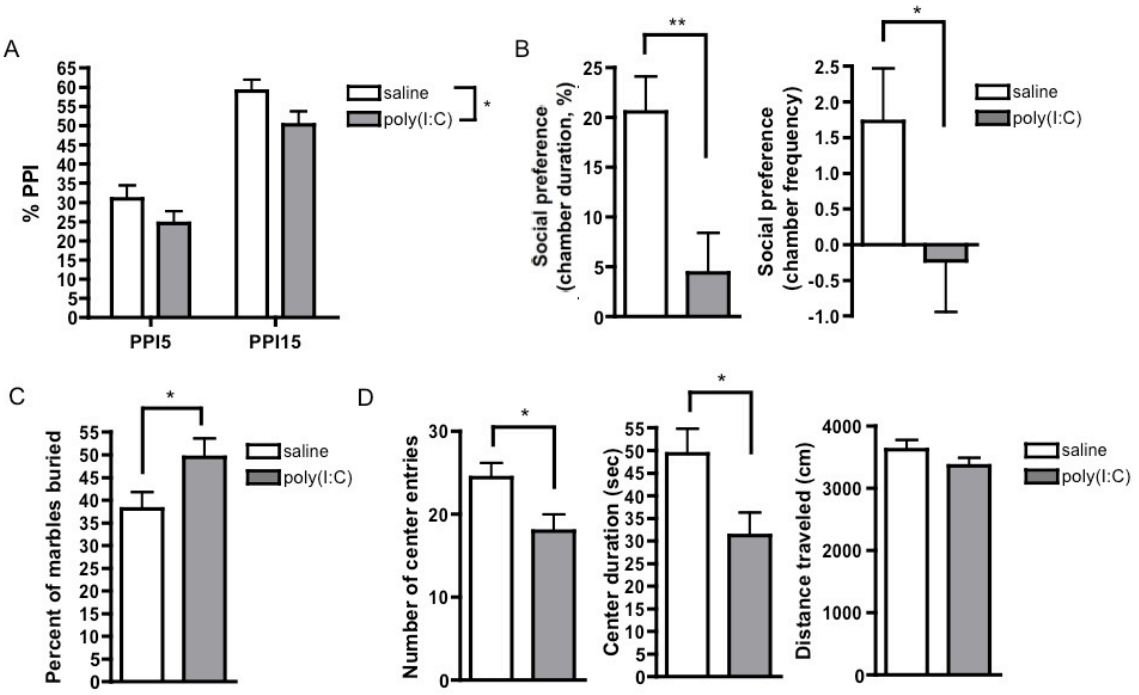

Fig. S6. BM transplant recipients are validated to be behaviorally abnormal in PPI, social preference, repetitive marble burying, and open-field exploration. (A) MIA offspring display significantly decreased PPI when prepulses are administered at 5 dB and 15 dB above background (PPI5 and PPI15, respectively). (B) Offspring of poly(I:C)-injected mothers exhibit decreased social preference for a novel mouse over a familiar mouse, as measured by differences in chamber duration (Left) and chamber frequency (Right). (C) MIA offspring bury significantly greater numbers of marbles compared with saline controls, pre-BM transplant. (D) Poly(I:C) offspring exhibit increased anxiety in the open field compared with saline controls, as measured by decreased entries into and duration in the center of the arena, and no significant difference in overall locomotion as denoted by total distance traveled (n = 20–21). *P < 0.05, **P < 0.01. All panels represent one representative experiment of at least two separate trials. BM transplant data were acquired from one large experiment.

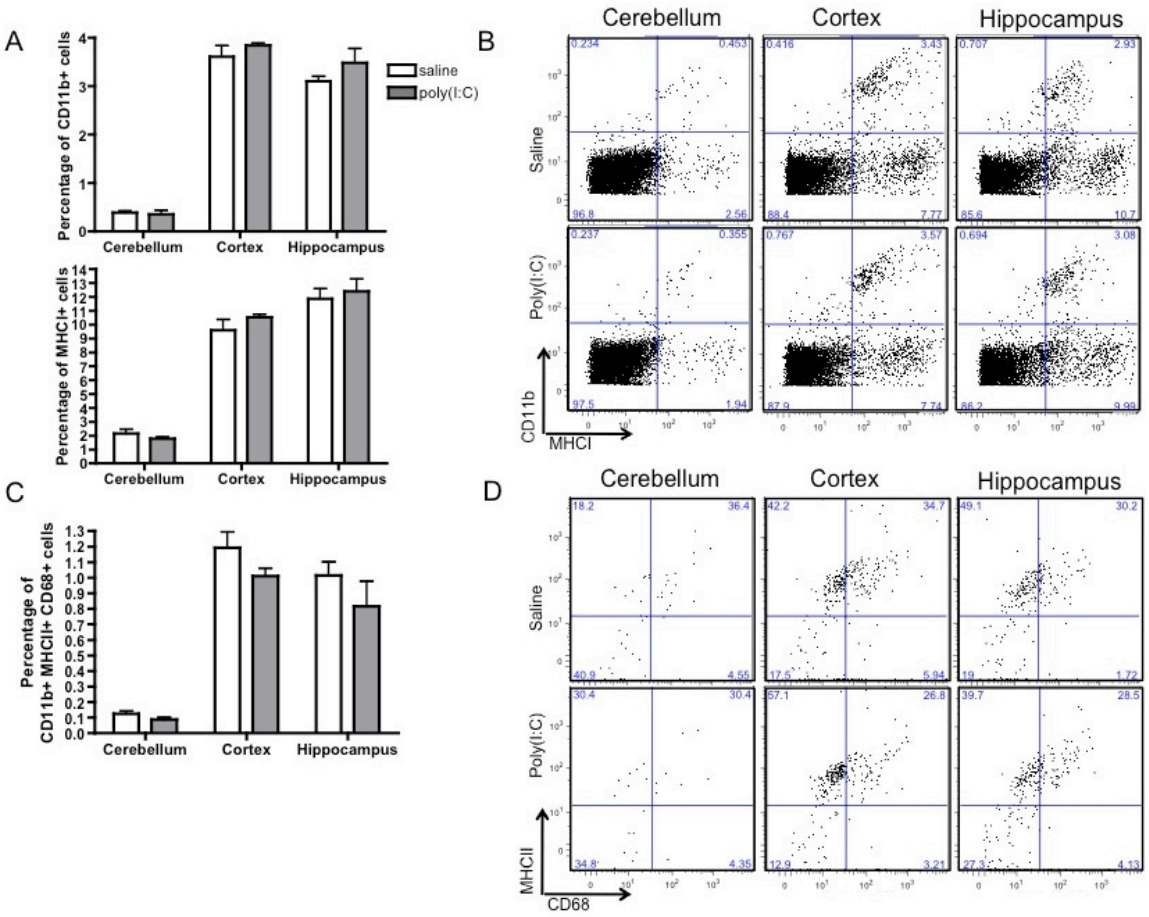

Fig. S7. MIA does not affect levels of activated or total CD11b+ microglia/macrophages in the brains of adult offspring. (A) There is no significant difference in levels of total CD11b+ cells or MHCI+ cells in the cerebellum, cortex, or hippocampus of adult poly(I:C) versus saline mice. (B) Representative flow cytometry plots of the separation of brain CD11b+ and MHCI+ cells. (C) There is no significant difference in the percentage of CD11b+ MHCII+ CD68+ cells from the cerebellum, cortex, or hippocampus of adult poly(I:C) versus saline mice. (D) Representative flow cytometry plots of the separation of brain MHCII+ and CD68+ cells. (n = 4 for each brain region).

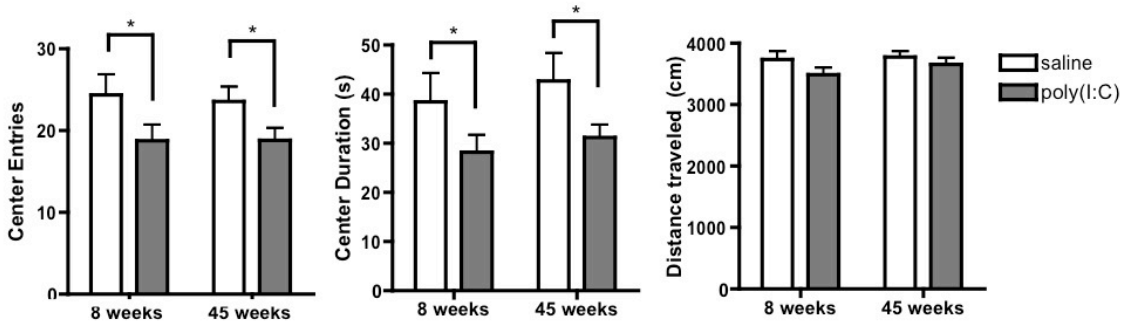

Fig. S8. MIA offspring retested for anxiety in the open field at 9 mo of age retain behavioral deficits of similar intensity to those seen at 2 mo of age. This result applies to the number of entries into the center of the open field (Left) and duration of time spent in the center of the open field (Center). No significant difference is observed between saline and poly(I:C) offspring in total distance traveled in the open field arenas (Right), as is typically observed in the MIA model (n = 17–19). *P < 0.05

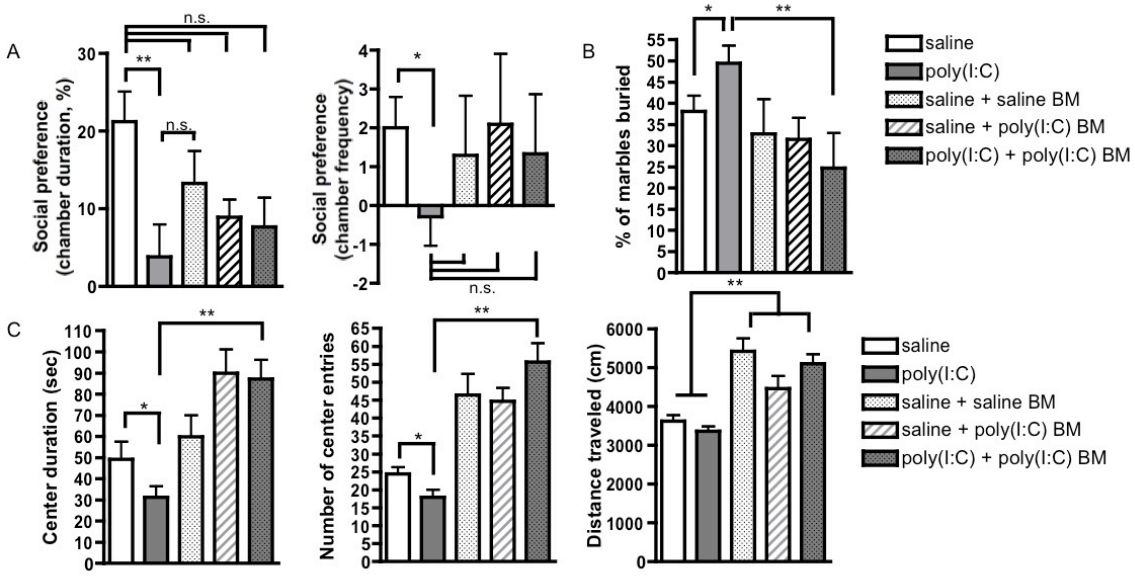

Fig. S9. The abnormal behaviors of MIA offspring are not maintained after irradiation and BM transplant. (A) Saline and poly(I:C) offspring irradiated and transplanted with poly(I:C) BM exhibit no difference in social preference for a novel mouse over a familiar mouse, as indicated by chamber duration (Left) and chamber frequency (Right). (B) MIA and control offspring irradiated and transplanted with poly(I:C) BM exhibit levels of repetitive marble burying that are comparable to those observed in transplanted control and untransplanted saline offspring. (C) There is no significant difference in level of anxiety in the open field between saline and poly(I:C) offspring irradiated and transplanted with saline or poly(I:C) BM, as measured by duration spent in the center of the open arena (Left) and number of entries into the center arena (Center). However, irradiated and BM-transplanted mice exhibit increased overall activity in the open field compared with untreated mice, as seen in elevated total distance traveled (Right). [n = 11–16 animals for saline and poly(I:C) groups, 4–5 animals for BM-transplant groups]. *P < 0.05, **P < 0.01, n.s., not significant. BM-transplant data were acquired from one large experiment.

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Chapter 12

A commensal bacterium of the gut microbiome modulates serum metabolites and ameliorates behavioral abnormalities in a mouse model of an autism risk factor

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Abstract

Although autism spectrum disorder (ASD) is defined by core behavioral impairments, gastrointestinal (GI) symptoms are commonly reported. Subsets of ASD individuals display dysbiosis of the gut microbiome, and some exhibit increased intestinal permeability. Here we demonstrate GI barrier defects in a mouse model of an important ASD risk factor, maternal immune activation (MIA). Remarkably, oral treatment of MIA offspring with the human commensal *Bacteroides fragilis* corrects gut permeability and ameliorates defects in communicative, stereotypic, anxiety-like and sensorimotor behaviors. MIA offspring also display an altered serum metabolomic profile, and *B. fragilis* modulates levels of several of the serum metabolites. We show that treating naïve mice with a metabolite that is induced by MIA and restored by *B. fragilis* treatment causes some behavioral abnormalities, suggesting that repair of gut permeability ameliorates behavior. Taken together, these findings support a gut-microbiome-brain connection in autism, and identify a potential probiotic therapy for ASD.

Introduction

Autism is a serious neurodevelopmental disorder characterized by stereotypic behavior and deficits in language and social interaction. The incidence of autism has rapidly increased to 1 in 88 births in the United States(CDC, 2012), representing a significant medical and social burden in the coming decades. However, therapies for treating the core symptoms of autism are limited, and reproducible molecular diagnostics have not been developed. Much research into autism spectrum disorder (ASD) has focused on genetic, behavioral and neurological aspects of the illness, but primary roles for environmental risk factors(Hallmayer et al., 2011), immune dysregulation(Onore, Careaga, & Ashwood, 2012) and additional peripheral disruptions(Kohane et al., 2012) in the pathogenesis of ASD have recently gained significant attention. Of several potential contributions to ASD, gastrointestinal (GI) distress is of particular interest, given its prevalence and correlation with the severity of core autism behavioral abnormalities(Adams, Johansen, Powell, Quig, & Rubin, 2011; Buie et al., 2010). A significant subset of ASD children display GI abnormalities, including abdominal cramps, chronic diarrhea or constipation and increased intestinal permeability(D'Eufemia et al., 1996; de Magistris et al., 2010). Moreover, antibiotic treatment and restricted diet are reported to provide behavioral improvements for some autistic children(Buie et al., 2010). The causes of these GI problems are unclear, but may be linked to gut bacteria, as the intestinal microbiome is altered in ASD individuals(Finegold, Downes, & Summanen, 2012; Williams, Hornig, Parekh, & Lipkin, 2012).

Humans are colonized with a diverse gut microbiota, which plays a critical role in obesity and inflammatory bowel disease (IBD)(Kau, Ahern, Griffin, Goodman, &

Gordon, 2011). In animal models, the microbiota not only modulates the development and function of the enteric immune system, but also impacts neuroinflammation(Hooper, Littman, & Macpherson, 2012; Ochoa-Reparaz et al., 2010). In particular, the human commensal *Bacteroides fragilis* exhibits therapeutic properties in mouse models of both colitis and multiple sclerosis (MS)(Ochoa-Reparaz et al., 2010; Round & Mazmanian, 2010). Recent studies have revealed that commensal bacteria also affect a variety of behaviors, including emotional, nociceptive and anxiety-like behaviors(Amaral et al., 2008; Bravo et al., 2011; Heijtz et al., 2011), and contribute to brain development and function in mice(Al-Asmakh, Anuar, Zadjali, Rafter, & Pettersson, 2012; Collins, Surette, & Bercik, 2012; Cryan & Dinan, 2012). Based on an emerging appreciation of a gut-brain connection(Mayer, 2011), we asked whether commensal bacteria, and *B. fragilis* in particular, could affect behaviors relevant to ASD.

Maternal immune activation (MIA) is an important environmental risk factor for ASD. Several large epidemiological studies link maternal viral and bacterial infection with increased autism risk in the offspring(Atladottir et al., 2010; Lee et al., 2012). We have shown that modeling this risk factor in mice by injecting pregnant dams with the viral mimic poly(I:C) yields offspring that exhibit the core behavioral symptoms of autism, as well as a common autism neuropathology(Malkova, Yu, Hsiao, Moore, & Patterson, 2012; Shi et al., 2009). MIA offspring also display altered peripheral immune responses(Hsiao & Patterson, 2011), which aligns well with recent studies highlighting a role for immune dysregulation in ASD(Onore et al., 2012). Although several environmental and genetic risk factors have been investigated in mice, GI abnormalities have not been reported in preclinical models of ASD. We show herein that MIA offspring

have defects in intestinal integrity and that treatment of MIA offspring with *B. fragilis* improves gut barrier function and ameliorates autism-related behaviors. Consistent with the finding that gut bacteria impact the serum metabolome(Bercik et al., 2011; Nicholson et al., 2012), *B. fragilis* treatment restores levels of specific serum metabolites that are altered by prior MIA exposure. Injection of the most prominently affected metabolite into naïve mice causes a behavioral impairment related to ASD. Thus, modulating serum metabolites by *B. fragilis* provides a potential pathway for its beneficial effects and may represent a novel approach for treatment of human autism.

B. fragilis treatment ameliorates ASD-related behaviors

As previously reported(Malkova et al., 2012), maternal poly(I:C) injection yields offspring with increased anxiety in the open field, increased stereotyped marble burying, decreased number and duration of ultrasonic vocalizations, deficient prepulse inhibition (PPI) of acoustic startle, and low sociability and social preference (Fig. 1; compare saline (S) to poly(I:C) (P)). Remarkably, oral treatment with *B. fragilis* ameliorates many of these ASD-associated behavioral abnormalities in MIA offspring. Probiotic-treated poly(I:C) offspring are protected from anxiety-like behavior in the open field (Fig. 1a) and also exhibit significantly decreased levels of stereotyped marble burying and restored number of ultrasonic vocalizations, with the duration per call exceeding that observed in control offspring (Fig. 1b, d; compare poly(I:C) (P) to poly(I:C)+*B. fragilis* (P+BF)). *B. fragilis* treatment also improves sensorimotor gating as indicated by the restoration of PPI to levels comparable to controls, with no significant effect on the intensity of startle to the acoustic stimulus (Fig. 1c). Interestingly, behavioral improvement in response to *B.*

fragilis treatment is not associated with changes in systemic immunity in MIA offspring (Supplementary Fig. 1) and is not dependent on polysaccharide A (PSA), the capsular component identified to confer immunomodulatory effects of *B. fragilis* in experimental autoimmune encephalomyelitis (EAE) and colitic mice (Supplementary Fig. 2)(Ochoa-Reparaz et al., 2010; Round & Mazmanian, 2010). Furthermore, amelioration of behavior is not specific to *B. fragilis*, as similar treatment with *Bacteroides thetaiotaomicron* also significantly improves anxiety-like, repetitive and communicative behavior in MIA offspring (Supplementary Fig. 3). While we cannot exclude the possibility of a tissue-specific effect of *B. fragilis* on immunity, these data suggest a novel beneficial effect of *B. fragilis* in the autism model as compared to models of MS or IBD. Notably, *B. fragilis* does not significantly alter baseline behavior in saline offspring (Fig. 1; compare saline (S) to saline+*B. fragilis* (S+BF)) in all tests but PPI, suggesting that the diseased state elicits a physiological response to *B. fragilis* that differs from that found in treated control mice.

Although *B. fragilis*-treated poly(I:C) offspring exhibit improved communicative, repetitive, anxiety-like and sensorimotor behavior, they retain deficits in sociability, as indicated by decreased preference for a novel mouse versus a novel toy, and in social preference for an unfamiliar versus familiar mouse (Fig. 1e). Interestingly, this parallels the inability to improve social behavior by administration of risperidone to ASD individuals(Canitano & Scandurra, 2008) or CNTNAP2 knockout mice, a genetic mouse model for ASD(Penagarikano et al., 2011), and by irradiation and bone marrow transplant of MIA offspring(Hsiao & Patterson, 2011). These data suggest that there are fundamental differences in the circuitry or circuit plasticity governing social behavior as

compared to the other behaviors, and that *B. fragilis* modulates specific pathways during amelioration of ASD-related behavioral defects in MIA offspring.

B. fragilis improves intestinal barrier integrity in offspring of immune-activated mothers

There is growing evidence that subsets of autistic children display GI abnormalities, including increased intestinal permeability or "leaky gut"(Buie et al., 2010; Ibrahim, Voigt, Katusic, Weaver, & Barbaresi, 2009). Remarkably, we find that MIA offspring display a significant deficit in intestinal barrier permeability, as reflected by increased translocation of orally administered FITC-dextran across the intestinal epithelial layer and into the circulation (Fig. 2a, left panel). A similar deficit is present in 3-week-old MIA offspring (Fig. 2a, right panel), demonstrating that this abnormality is established early during development and prior to *B. fragilis* treatment. Notably, *B. fragilis* treatment restores this MIA-associated increase in intestinal barrier permeability to levels comparable to those observed in saline controls (Fig. 2a, left panel). These data demonstrate that, in addition to improving autism-related behaviors, *B. fragilis* ameliorates leaky gut in MIA offspring. The presence of GI defects prior to probiotic administration suggests that *B. fragilis* may treat, rather than prevent, this ASD-related pathology in MIA offspring.

To assess the molecular basis for increased intestinal permeability in MIA offspring, we examined the colons of MIA offspring for levels of the tight junction components ZO-1, ZO-2, ZO-3, occludin and claudins 1, 2, 3, 4, 7, 8, 12, 13 and 15(Holmes, Van Itallie, Rasmussen, & Anderson, 2006). Consistent with the leaky gut

phenotype, colons from adult poly(I:C) offspring exhibit decreased expression of ZO-1, ZO-2, occludin and claudin 8, and increased expression of claudin 15 mRNA (Fig. 2b). Furthermore, *B. fragilis* treatment ameliorates MIA-associated changes in expression of claudin 8 and 15, but has no restorative effect on expression of ZO-1, ZO-2 or occludin mRNA. Similar changes in colon claudin 8 and 15, with restoration by *B. fragilis* treatment, are also observed at the protein level (Fig. 2c). Interestingly, both decreased claudin 8 and increased claudin 15 are associated with alterations in sodium paracellular permeability in the mouse intestine(Amasheh et al., 2009; Tamura et al., 2011), and sodium electrochemical gradients across the intestinal epithelia are known to regulate sodium-dependent ion and nutrient absorption as well as GI motility(Turner, 2009). No such effects of *B. fragilis* on tight junction expression are observed in small intestines from MIA offspring (Supplementary Fig. 4), consistent with reports showing that *B. fragilis* colonizes the colonic mucosa and that *Bacteroides* species are predominantly found in the colon(Huang, Lee, & Mazmanian, 2011). Collectively, discovery of GI defects in MIA offspring recapitulates intestinal co-morbidities found in subsets of ASD individuals, and correction of leaky gut and behavioral abnormalities by *B. fragilis* supports emerging evidence for a gut-brain link in autism.

B. fragilis **modulates an MIA-associated cytokine in the gut**

Cytokines are signaling molecules used widely by both the immune and nervous systems. Levels of a variety of cytokines, including interleukin-6 (IL-6), are altered in the serum and cerebrospinal fluid of autistic individuals(Onore et al., 2012; Patterson, 2011), but whether they also exhibit altered gut expression patterns is unknown. Remarkably,

colonic tissues of adult MIA offspring contain increased levels of IL-6 mRNA and protein, and *B. fragilis* treatment restores elevated IL-6 to levels found in control mice (Supplementary Fig. 5). Levels of other cytokines are also altered in both colons and small intestines of MIA offspring (Supplementary Fig. 4 and Supplementary Fig. 5), but these are not affected by *B. fragilis* treatment, revealing specificity for IL-6. This is interesting in light of a previous study showing that induction of IL-6, but not other cytokines, in response to MIA is required for the development of behavioral deficits in the offspring(S. E. Smith, Li, Garbett, Mirnics, & Patterson, 2007). Nonetheless, altered intestinal cytokines may be the basis for the increased intestinal permeability observed in MIA offspring, as several cytokines including IL-6 regulate tight junction expression and intestinal barrier integrity(Suzuki, Yoshinaga, & Tanabe, 2011; Turner, 2009). Consistent with the notion that IL-6 affects intestinal permeability in MIA offspring, we find that recombinant IL-6 treatment can modulate colon levels of both claudin 8 and claudin 15 in *in vivo* and in *in vitro* colon organ cultures (Supplementary Fig. 6). This suggests that *B. fragilis*-mediated restoration of colonic IL-6 levels regulates changes in colon tight junction protein expression. Overall, these findings demonstrate that MIA offspring exhibit defective GI barrier integrity, with corresponding changes in colon tight junction and cytokine profiles, which are reversed by *B. fragilis* treatment.

The serum metabolome is modulated by MIA and *B. fragilis*

Metabolomic studies have shown that gut microbial products are found in many extra-intestinal tissues, and molecules derived from the microbiota may influence metabolic, immunologic and behavioral endophenotypes in mice and humans(Bercik et

al., 2011; Blumberg & Powrie, 2012; Hooper et al., 2012; MacFabe, 2012; Matsumoto et al., 2012; Nicholson et al., 2012). Given that MIA offspring display altered intestinal permeability and tight junction expression, we utilized liquid chromatography/gas chromatography with mass spectrometry (LC/GC-MS)-based metabolomic profiling to identify MIA-associated changes in serum metabolites. 2400 metabolites were assayed and of these, 322 metabolites, spanning amino acid (94), peptide (15), carbohydrate (22), energy (10), lipid (128), nucleotide (23), xenobiotic (19) and cofactor and vitamin (11) super pathways were detected in sera from adult mice. Interestingly, MIA leads to statistically significant alterations in 8% of all serum metabolites detected (Supplementary Table 1). Furthermore, postnatal *B. fragilis* treatment has a significant effect on the serum metabolome, altering 34% of all metabolites detected (Supplementary Fig. 7 and Supplementary Data 1). The majority (72%) of *B. fragilis*-mediated changes are found in MIA offspring and not in control mice, demonstrating that the particular effects of postnatal *B. fragilis* treatment on the serum metabolome depend on the disease status of the host. This is consistent with our finding that *B. fragilis*-mediated improvement of many ASD-related behaviors is specific to MIA offspring and is not observed in controls (Fig. 1). In particular, *B. fragilis* treatment lowers serum levels of several fatty acids and increases levels of molecules associated with purine catabolism (Supplementary Fig. 7 and Supplementary Data 1). In light of our finding that *B. fragilis* itself does not permanently colonize the GI tracts of treated MIA and control offspring (Supplementary Fig. 8), these widespread changes suggest that *B. fragilis* disrupts the composition and/or functional activity of the resident intestinal microbiota. Moreover, that vehicle-treated MIA offspring do not display many of these changes in fatty acid and

purine metabolites suggests that these disruptions do not play a significant role in the onset of autism-related symptoms in the MIA model. Whether *B. fragilis*-mediated changes in fatty acid uptake and purine salvage serve a protective role in ameliorating MIA-induced autism-related behaviors remains to be investigated.

B. fragilis corrects levels of MIA-induced serum metabolites

In line with the notion that increased gut permeability leads to leakage of metabolites into the bloodstream, we hypothesized that *B. fragilis*-mediated improvement of intestinal barrier integrity prevents alterations in serum metabolite levels. We therefore focused on serum metabolites that are significantly altered by MIA treatment and completely restored to control levels by *B. fragilis* treatment (Fig. 3). MIA offspring display a striking, 45-fold increase in serum levels of a recently identified metabolite, 4-ethylphenylsulfate (4EPS), which is dramatically reduced by *B. fragilis* treatment (Fig. 3a). This metabolite is of particular interest because of the reported production of 4EPS by GI microbes and proposed role of 4EPS in communication by mice(Lafaye et al., 2004). Moreover, we find that, compared to conventionally colonized mice, germ-free mice display nearly undetectable levels of serum 4EPS, indicating that serum 4EPS is derived from or critically modulated by the commensal microbiota (Fig. 4e). Interestingly, 4EPS is suggested to be a uremic toxin, as is *p*-cresol (4-methylphenol), a metabolite identified as a possible urinary biomarker for human autism(Altieri et al., 2011). MIA offspring also exhibit elevated levels of serum *p*-cresol, though the increase does not reach statistical significance (Supplementary Data 1). That 4EPS shares such structural similarity to the toxic sulfated form of *p*-cresol (4-methylphenylsulfate) is

intriguing in its suggestion that the two metabolites may exhibit functional overlap and link metabolite abnormalities seen in the MIA model to those observed in human autism.

In addition to 4EPS, MIA offspring display significantly increased levels of serum indolepyruvate, a key molecule of the tryptophan metabolism pathway, which is restored to control levels by *B. fragilis* treatment (Fig. 3b). Indolepyruvate is generated by tryptophan catabolism and, like 4EPS, indolepyruvate is known to be produced by gut microbes(E. A. Smith & Macfarlane, 1997). Moreover, the elevation in serum indolepyruvate observed in MIA offspring is analogous to the increase in another major tryptophan metabolite observed in human autism, indolyl-3-acryloylglycine (IAG), which was suggested to be a urinary biomarker for ASD(Bull et al., 2003). Interestingly, IAG is involved in GI homeostasis and is produced by bacterial tryptophan metabolism(Keszthelyi, Troost, & Masclee, 2009). Although IAG was not detected in our metabolomic screen, it is notable that MIA offspring exhibit increased levels of serum serotonin ($0.05 < P < 0.10$; Supplementary Data 1), which reflects an alteration in another pathway of tryptophan metabolism and is reminiscent of the hyperserotonemia endophenotype of autism(Mulder et al., 2004). Importantly, the commensal microbiota is known to impact serum levels of indole-containing tryptophan metabolites and serotonin(Wikoff et al., 2009). MIA also leads to altered serum glycolate, imidazole propionate and N-acetylserine levels (Fig. 3c-e), which are corrected by *B. fragilis* treatment. How changes in these metabolites may be relevant to ASD or GI dysfunction, however, are unclear. Overall, we demonstrate that MIA elevates, and *B. fragilis*

treatment normalizes, serum levels of 4EPS and indolepyruvate, two molecules modulated by the intestinal microbiota with potential relevance to autism.

A serum metabolite induces some ASD-related behaviors

MIA-induced increases in the systemic bioavailability of specific metabolites and restoration by *B. fragilis* treatment suggests that these molecules may play a causative role in the development of autism-related behaviors in MIA offspring. To test this hypothesis, we examined whether experimental increase in serum 4EPS, the most dramatic of all MIA-induced and *B. fragilis*-restored metabolite changes, is sufficient to cause autism-related behavioral abnormalities in mice. We chemically synthesized 4EPS by treatment of 4-ethylphenol with sulfur trioxide-pyridine complex, which following ion exchange, provided the 4EPS potassium salt (Supplementary Fig. 9a)(Burlingham et al., 2003; Grimes, 1959). Mice were intraperitoneally treated with 4EPS daily, from 3 weeks of age (when increased intestinal permeability is detected in MIA offspring, Fig. 2a) to 6 weeks of age (when behavior testing begins). Remarkably, systemic administration of 4EPS to naïve mice is sufficient to induce anxiety-like behavior that is similar to that observed in MIA offspring (Fig. 4a). In addition, 4EPS-treated mice exhibit an abnormal PPI response characterized by increased startle to the primary acoustic stimulus (Fig. 4b). There are no significant differences, however, between 4EPS-treated versus saline-treated mice in marble burying or USV behavior (Fig. 4c, d), suggesting that elevating serum 4EPS levels specifically promotes anxiety-like behavior. It will be interesting to assess whether continued 4EPS administration through the behavioral testing period influences communicative and stereotyped behavior. More likely, complex behavioral conditions

may be mediated by combinations of serum metabolites, in addition to pathology unrelated to metabolome changes. In line with a GI involvement in modulating the serum metabolome and behavior in MIA offspring, we find that serum 4EPS levels are modulated by the commensal microbiota (Fig. 4e) and that elevating serum 4EPS in naïve mice is sufficient to induce anxiety-like behavior. This indicates that metabolomic changes can contribute to the onset and/or persistence of autism-related behavioral abnormalities in MIA offspring, and suggests that *B. fragilis*-mediated modulation of serum 4EPS levels leads to the correction of anxiety-like behavior in MIA offspring. Overall, our studies reveal that metabolomic changes may cause some ASD-related behavioral abnormalities in mouse models, suggesting that inhibition of specific serum metabolites represents a novel approach to autism therapies.

Conclusion

While the impact of the microbiota on immunologic and metabolic disease is profound, little is known regarding a link to behavioral disorders. We propose that commensal bacteria of the microbiota can influence the gut-brain connection by modulating metabolites that alter behavior. We find that postnatal *B. fragilis* treatment corrects abnormal intestinal permeability and ameliorates communicative, stereotyped, sensorimotor and anxiety-like behavior in a mouse model for ASD. While a number of microbes, including *Lactobacillis rhamnosus*(Bravo et al., 2011) and *Bifidobacterium longum(Bercik et al., 2011)*, are known to alleviate anxiety and depressive behavior in animal models, our current findings represent the first evaluation of a microbial effect on core autism-related behaviors. We further demonstrate that, in addition to displaying

cardinal behavioral and neuropathological symptoms of ASD(Malkova et al., 2012; Shi et al., 2009), offspring of immune-activated mothers exhibit altered serum metabolites and deficient GI integrity that is analogous to that seen in subsets of ASD individuals(Buie et al., 2010; D'Eufemia et al., 1996; de Magistris et al., 2010). Thus, the MIA model exhibits face and construct validity for particular co-morbid GI symptoms found in human autism. Consistent with the well-established role of GI microbes in regulating intestinal permeability and metabolic homeostasis(Ewaschuk et al., 2008; Matsumoto et al., 2012; Nicholson et al., 2012; Resta-Lenert & Barrett, 2009; Turner, 2009; Wikoff et al., 2009), we show that *B. fragilis* treatment corrects GI permeability and restores MIA-associated changes in blood metabolites. By this means, *B. fragilis* may prevent the leakage of deleterious molecules from the GI lumen and/or promote the synthesis of protective compounds in the periphery. In a proof-of-concept test of this idea, the microbially-modulated metabolite 4EPS, which is elevated in circulating blood by MIA and restored by *B. fragilis* treatment, is sufficient to induce anxiety-like behavior in wildtype mice. Although core autism behaviors are not affected by 4EPS alone, this finding warrants the study of several other blood metabolites affected by *B. fragilis* treatment for their potential to impact particular autism-related behaviors. It is intriguing that *B. fragilis* exerts beneficial behavioral and metabolomic effects in a disease-specific manner. This observation contributes to a growing number of studies emphasizing that microbial composition and function is critically influenced by differences in host genotype and environment(Campbell et al., 2012; Gulati et al., 2012; P. Smith et al., 2012). Taken together, we provide a novel mechanism by which *B. fragilis* treatment can improve autism-related behavioral abnormalities and present compelling evidence for a

probiotic-based therapy for ASD-associated symptoms. Validation of a specific metabolomic profile specific in human autism subjects with GI complications may serve as a novel molecular diagnostic and the basis for microbiome-mediated therapies.

Methods Summary

Maternal immune activation was induced by injection of 20 mg/kg poly(I:C) sodium salt (Sigma Aldrich) into pregnant E12.5 C57Bl/6N females. Offspring were treated with three doses of 10^10 cfu *B. fragilis* in applesauce or 1.5% sodium bicarbonate vehicle in applesauce at weaning. Mice were behaviorally tested from 6-10 weeks of age in prepulse inhibition, open field exploration, repetitive marble burying, sociability, social preference and adult ultrasonic vocalization. *In vivo* intestinal permeability assays were conducted by oral gavage with 4kD FITC-dextran (Sigma Aldrich) and serum fluorescence detection at 521 nm using an xFluor4 spectrometer (Tecan). Colon lysates from adult mice were used for 20-plex cytokine arrays (Luminex) or processed for gene expression analysis by RNA extraction with Trizol (Invitrogen) and RNeasy mini column isolation (Qiagen) with DNase treatment (Qiagen), cDNA synthesis with iScript kit (Biorad) and SYBR Green qRT-PCR with Rox passive reference dye (Roche) using tight junction and cytokine-related primers. Metabolomics screens were conducted by GC/LC-MS (Metabolon Inc.) using a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear ion-trap front-end and a Fourier transform ion cyclotron resonance mass spectrometer back-end. 4EPS was measured by LC-MS at Caltech using an Agilent 110 Series HPLC system equipped with a photodiode array detector and interfaced to a model G1946C single quadrupole expectospray mass spectrometer.

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Author Contributions

E.Y.H, P.H.P and S.K.M. designed the study, E.Y.H., S.W.M., S.H., and J.A.C. and J.C. performed the experiments and analyzed the data, E.Y.H., P.H.P. and S.K.M. wrote the manuscript. P.H.P. and S.K.M contributed equally to the study. All authors discussed the results and commented on the manuscript.

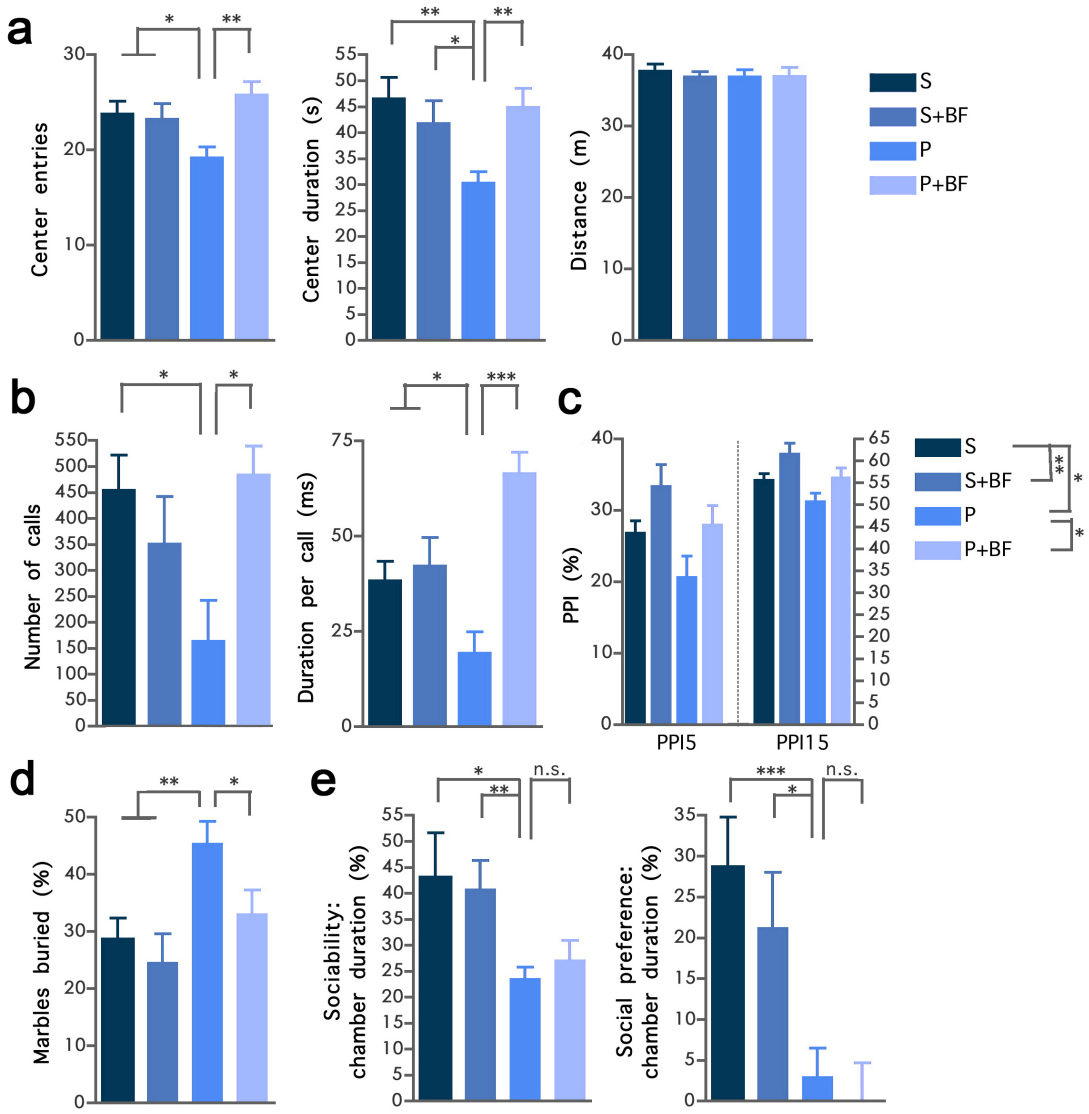


Figure 1. *B. fragilis* treatment ameliorates autism-related behaviors in MIA offspring. a, Poly(I:C) offspring exhibit anxiety-like behavior, as measured by decreased entries into and duration spent in the center of an open field, which is restored to control levels by *B. fragilis* treatment. There is no difference between experimental groups in total distance traveled. n = 35-75. **b,** *B. fragilis* treatment corrects deficits in communicative behavior, as indicated by the total number and duration of ultrasonic vocalizations produced by adult male poly(I:C) offspring in response to a female. n = 7-10. **c,** *B. fragilis*-treated poly(I:C) and saline offspring display increased prepulse

inhibition (PPI) of an acoustic startle, indicating improved sensorimotor gating (PPI5, 5 db prepulse; PPI15, 15 db prepulse). n = 35-75. **d,** Poly(I:C) offspring engage in elevated stereotypic marble burying, and this phenotype is corrected by *B. fragilis* treatment. n = 16-45. **e,** *B. fragilis* is not effective in treating social abnormalities in MIA offspring, having no impact on the decreased preference for a novel mouse versus novel toy (sociability) or the decreased preference for an unfamiliar versus familiar mouse (social preference) observed in MIA offspring. n = 10. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, n.s. = not significant. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis*

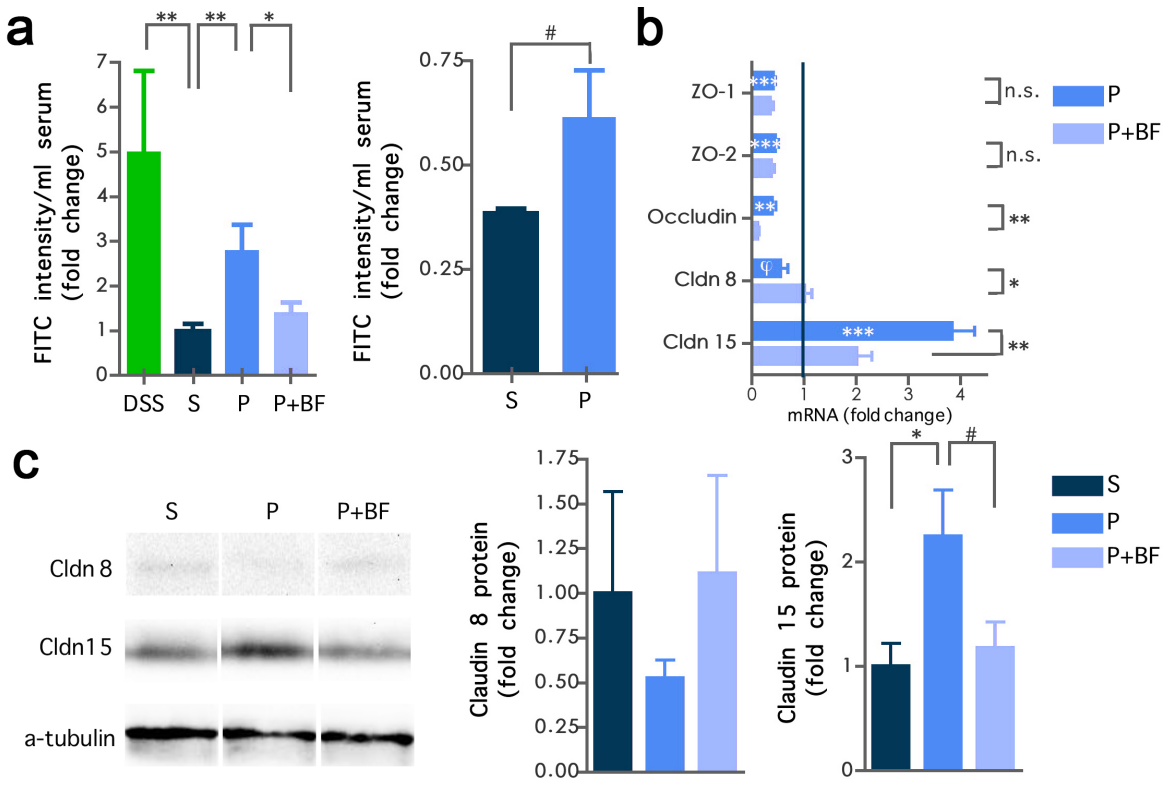


Figure 2. MIA offspring exhibit a deficit in GI barrier function and expression of tight junction components, which are corrected by *B. fragilis* treatment. a, *Left panel:* Adult poly(I:C) offspring display increased serum FITC fluorescence intensity after oral FITC-dextran gavage, indicating elevated intestinal permeability. This is restored to levels comparable to saline controls by *B. fragilis* treatment. DSS-treated colitic mice are used as a positive control for leaky gut. n= 12-13 for poly(I:C) and saline, n = 6 for DSS and poly(I:C)+BF. *Right panel:* 3 week old MIA offspring also exhibit increased intestinal permeability, demonstrating that this GI barrier abnormality is present prior to probiotic treatment. n = 4. **b,** The abnormal intestinal barrier integrity in MIA offspring corresponds to altered colon tight junction expression of ZO-1, ZO-2, occludin, claudin 8 and 15 mRNA. The changes in claudin 8 and 15 expression are ameliorated by *B. fragilis* treatment. n = 8. Asterisks within bars indicate significance

compared to saline control (normalized to 1, as denoted by the black line), whereas asterisks to the right of bars denote significance between poly(I:C) and poly(I:C)+*B. fragilis.* **c,** Similar alterations in colon claudin 8 and 15 are seen at the protein level, with trending decreases in claudin 8 and significant increases in claudin 15, which are both corrected by *B. fragilis* treatment. n = 3. # $P < 0.07$, φ $P = 0.05$, *$P < 0.05$, **$P < 0.01$, n.s. = not significant. DSS=dextran sodium sulfate, S=saline+vehicle, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis.*

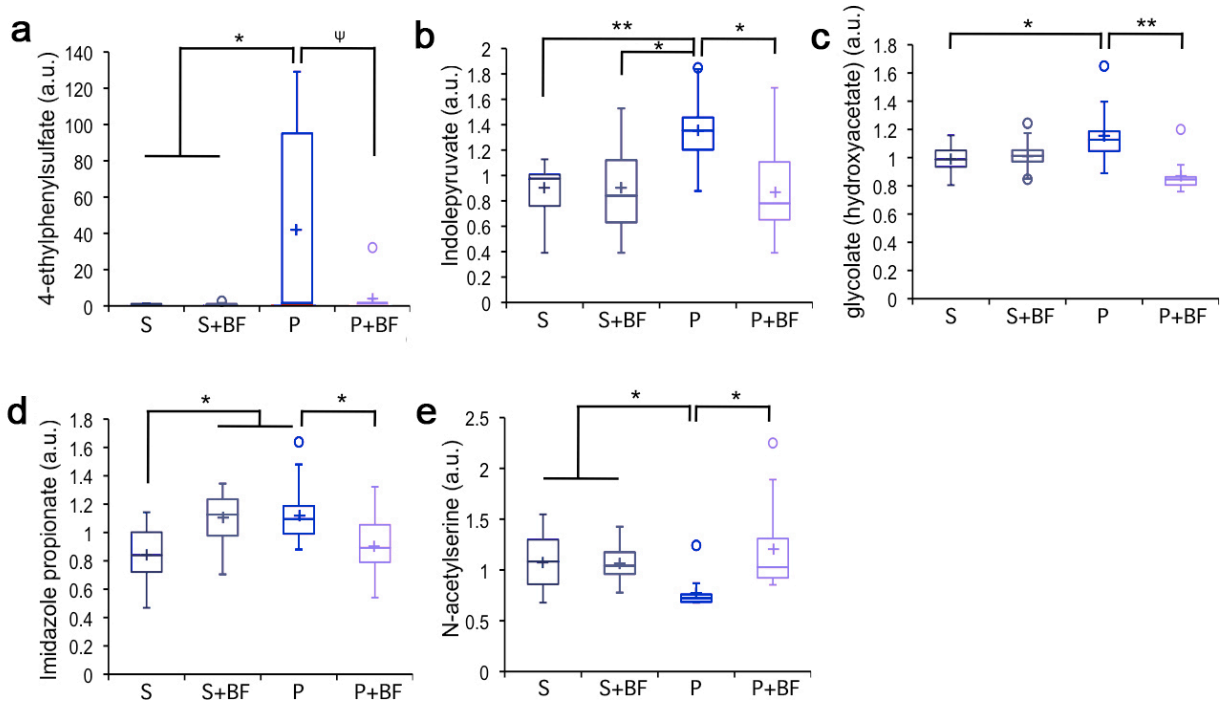


Figure 3. *B. fragilis* treatment corrects MIA-induced alterations in serum metabolites. a, Adult MIA offspring display a striking 45-fold increase in serum 4-ethylphenylsulfate levels, which is reduced to levels comparable to controls by *B. fragilis* treatment. MIA offspring also exhibit significantly increased serum indolepyruvate **(b)**, glycolate **(c)** and imidazole propionate **(d)** levels, and decreased serum N-acetylserine levels **(e)**, which are each corrected by *B. fragilis* treatment. n = 8. Ψ P =0.08, *$P < 0.05$, **$P < 0.01$, a.u.=relative arbitrary units. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis*.

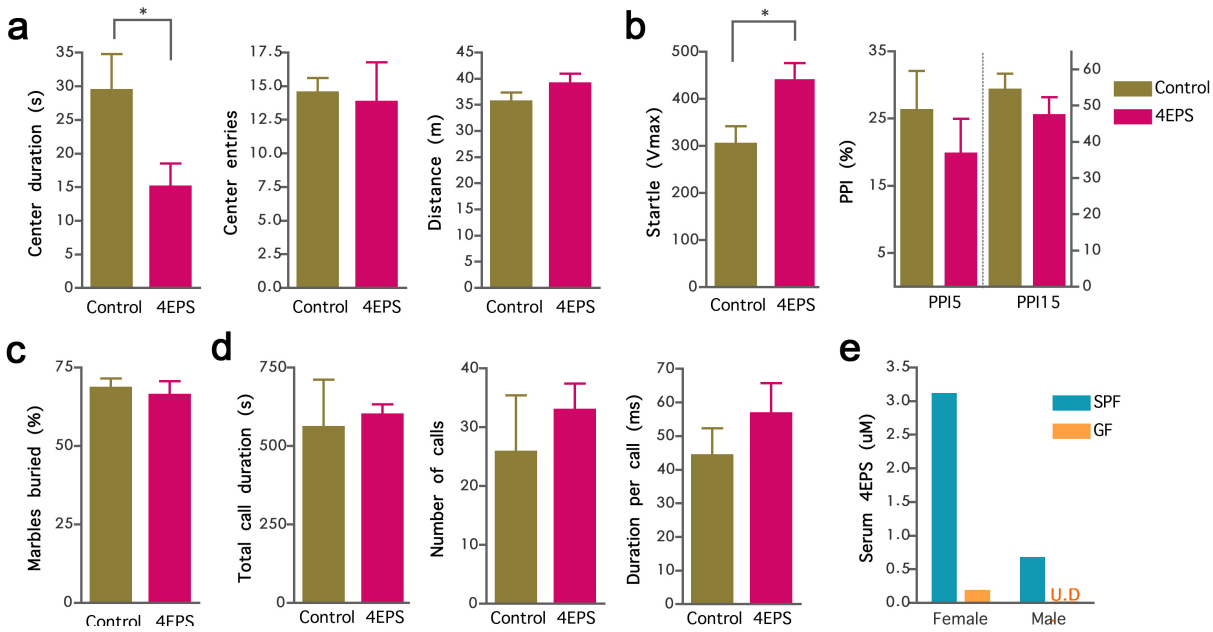


Figure 4. Elevating serum 4-ethylphenylsulfate (4EPS) levels is sufficient to induce anxiety-like behavior. a, 4EPS-treated mice exhibit anxiety-like behavior in the open field, as indicated by significantly decreased duration spent in the center arena, but no difference in total distance traveled, compared to saline-treated control mice. n = 10. **b,** 4EPS treatment also induces an altered PPI response characterized by significantly elevated startle to the 120 db acoustic tone, despite no significant difference in percent PPI. There is no significant effect of 4EPS treatment in the marble burying task **(c)** and in adult male ultrasonic vocalizations in the response to a female **(d)**. n = 10 (n = 5 males for USV). **e,** Germ-free (GF) mice display dramatically reduced levels of serum 4EPS compared to conventional specific-pathogen-free (SPF) controls, demonstrating that the commensal microbiota modulates serum 4EPS levels. UD=undetectable. n=1, where each represents pooled sera from 3-5 mice. *$P < 0.05$

Methods

Animals and MIA

Pregnant C57BL/6N (Charles River; Wilmington, MA) were injected i.p. on E12.5 with saline or 20 mg/kg poly(I:C) according to methods described in ref. (Hsiao & Patterson, 2011). All animal experiments were approved by the Caltech IACUC.

B. fragilis *treatment*

At 3 weeks of age, saline and poly(I:C) offspring were weaned and treated with *B. fragilis* freshly prepared every other day for 3 days: 10^10 cfu *B. fragilis* was mixed with 4 ml sugar-free applesauce and spread over four standard food pellets. Control saline and poly(I:C) animals were fed 1.5% sodium bicarbonate vehicle in applesauce over food pellets. Applesauce and pellets were completely consumed by mice of each treatment group by 48 hours after administration. The same procedure was used for treatment with mutant *B. fragilis* lacking PSA and *B. thetaiotaomicron*.

Behavioral testing

Adult MIA and control offspring were behaviorally tested as in refs. (Hsiao & Patterson, 2011) and (Malkova et al., 2012). Mice were tested beginning at 6 weeks of age for prepulse inhibition, open field exploration, marble burying, social interaction and adult ultrasonic vocalizations, in that order with at least 5 days between behavioral tests. Behavioral data for *B. fragilis* treatment and control groups (Fig. 1) represent cumulative results collected from multiple litters of 3-5 independent cohorts of mice for PPI and open field tests, 2-4 cohorts for marble burying, 2 cohorts for adult male ultrasonic

vocalization and 1 cohort for social interaction. Discrepancies in sample size across behavioral tests reflect differences in when a particular test was implemented.

Prepulse inhibition. PPI tests are used as a measure of sensorimotor gating and were conducted and analyzed as in refs (S. E. Smith et al., 2007) and (Geyer & Swerdlow, 2001). Briefly, mice were acclimated to the testing chambers of the SR-LAB startle response system (San Diego Instruments) for 5 min, presented with six 120 db pulses of white noise (startle stimulus) and then subjected to 14 randomized blocks of either no startle, startle stimulus only, 5 db prepulse with startle or 15 db prepulse with startle. The startle response was recorded by a pliezo-electric sensor, and the percent PPI is defined as [((startle stimulus only – 5 or 15 db prepulse with startle)/startle stimulus only)*100].

Open field exploration. The open field test is widely used to measure anxiety-like and locomotor behavior in rodents (Bailey & Crawley, 2009). Mice were placed in 50 x 50 cm white Plexiglas boxes for 10 min. An overhead video camera recorded the session, and Ethovision software (Noldus) was used to analyze the distance traveled, and the number of entries and duration of time spent in the center arena (central 17 cm square).

Marble burying. Marble burying is an elicited repetitive behavior in rodents analogous to those observed in autistic individuals(Silverman, Yang, Lord, & Crawley, 2010). This test was conducted and analyzed according to methods described in refs. (Thomas et al., 2009) and (Malkova et al., 2012). Mice were habituated for 10 min to a novel testing cage containing a 4 cm layer of chipped cedar wood bedding and then transferred to a new housing cage. 18 glass marbles (15 mm diameter) were aligned

equidistantly 6 x 3 in the testing cage. Mice were returned to the testing cage and the number of marbles buried in 10 min was recorded.

Sociability and social preference. Social interaction tests were conducted and analyzed according to methods adopted from refs. (Sankoorikal, Kaercher, Boon, Lee, & Brodkin, 2006) and (Yang, Silverman, & Crawley, 2011). Briefly, testing mice were habituated for 10 min to a 40 x 60 cm Plexiglas three-chambered apparatus containing clear interaction cylinders in each of the side chambers. Sociability was tested in the following 10 min session, where the testing mouse was given the opportunity to explore a novel same-sex, age-matched mouse in one interaction cylinder (social object) versus a novel toy (green sticky ball) in the other interaction cylinder of the opposite chamber. Social preference was tested in the final 10 min session, where the testing mouse was given the opportunity to explore a now familiar mouse (stimulus mouse from the previous sociability session) versus a novel unfamiliar same-sex, age-matched mouse. In each session, the trajectory of the testing mouse was tracked with Ethovision software (Noldus). Sociability data is presented as preference for the mouse over the toy: percent of time in the social chamber percent of time in the nonsocial chamber, and social preference data is presented as preference for the unfamiliar mouse over the familiar mouse: percent of time in the unfamiliar mouse chamber – percent of time in the familiar mouse chamber. Similar indexes were measured for chamber entries, and entries into and duration spent in the contact zone (7 x 7 cm square surrounding the interaction cylinder).

Adult ultrasonic vocalizations. Male mice produce USVs in response to female mice as an important form of communication(Portfors, 2007). We measured adult male USV production in response to novel female exposure according to methods described in

refs. (Grimsley, Monaghan, & Wenstrup, 2011; Malkova et al., 2012; Scattoni, Ricceri, & Crawley, 2011). Adult males were single-housed one week before testing and exposed for 20 min to an unfamiliar adult female mouse each day starting four days prior to testing in order to provide a standardized history of sexual experience and to adjust for differences in social dominance. On testing day, mice were habituated to a novel cage for 10 minutes before exposure to a novel age-matched female. USVs were recorded for 3 min using the UltraSoundGate microphone and audio system (Avisoft Bioacoustics). Recordings were analyzed using Avisoft's SASLab Pro software after fast Fourier transformation at 512 FFT-length and detection by a threshold-based algorithm with 5 ms hold time. Data presented reflect duration and number of calls produced in the 3 min session.

In vitro *immune assays*

Methods for Treg and Gr-1 flow cytometry and CD4+ T cell *in vitro* stimulation are described in ref. (Hsiao, McBride, Chow, Mazmanian, & Patterson, 2012). Briefly, cells were harvested in complete RPMI from spleens and mesenteric lymph nodes. For subtyping of splenocytes, cells were stained with Gr-1 APC, CD11b-PE, CD4-FITC and Ter119-PerCP-Cy5.5 (Biolegend). For detection of Tregs, splenocytes were stimulated for 4 h with PMA/ionomycin in the presence of GolgiPLUG (BD Biosciences), blocked for Fc receptors and labeled with CD4-FITC, CD25-PE, Foxp3-APC and Ter119-PerCP-Cy5.5. Samples were processed using the FACSCalibur cytometer (BD Biosciences) and analyzed using FlowJo software (TreeStar). For CD4+ T cell stimulation assays, 10^6 CD4+ T cells were cultured in complete RPMI with PMA (50 ng/ml) and ionomycin (750 ng/ml) for 3 d at 37C with 5% (vol/vol) CO2. Each day, supernatant was collected

for ELISA assays to detect IL-6 and IL-17, according to the manufacturer's instructions (eBioscience).

Intestinal permeability assay

Adult mice were fasted for 4 hours before oral gavage with 0.6 g/kg 4 kDa FITC-dextran (Sigma Aldrich). 4 hours later, blood samples were collected by cardiac puncture and spun through SST vacutainers (Becton Dickinson). FITC-dextran standards and 3X-diluted sera were immediately read for FITC fluorescence intensity at 521 nm using an xFluor4 spectrometer (Tecan). Mice were fed 3% dextran sulfate sodium salt (DSS; MP Biomedicals) in drinking water for 7 days to chemically induce colitis.

Intestinal qRT-PCR, Western blots, and cytokine profiles

1 cm regions of the distal, medial and proximal colon and small intestine were washed in HBSS and either a) homogenized in ice-cold Trizol for RNA isolation and reverse transcription according to ref. (Hsiao & Patterson, 2011) or b) homogenized in Tissue Extraction Reagent I (Invitrogen) containing EDTA-free protease inhibitors (Roche) for protein assays. For SYBR green qRT-PCR, validated primer sets were obtained from Primerbank (Harvard). For cytokine profiling, mouse 20-plex cytokine arrays (Invitrogen) were run on the Luminex FLEXMAP 3D platform by the Clinical Immunobiology Correlative Studies Laboratory at the City of Hope (Duarte, CA). Western blots were conducted according to standard methods and probed with rabbit anti-claudin 8 or rabbit anti-claudin 15 (Invitrogen) at 1:88 dilution.

IL-6 oral gavage and in vitro colon culture

For *in vivo* assays, adult mice were gavaged with 5 ug carrier-free recombinant mouse IL-6 (eBioscience) in 1.5% sodium bicarbonate. At 4 hours post-gavage, 1 cm regions of distal, medial and proximal colon were dissected, washed in HBSS and processed for qRT-PCR, as described above. For *in vitro* assays, adult mice were sacrificed and 1 cm regions of distal, medial and proximal colon were dissected, washed and bisected for colon culture with 0-80 ng/ml IL-6 in complete RPMI media. After 4 hours of culture, colon pieces were harvested and processed for qRT-PCR, as described above.

B. fragilis *colonization assay*

Fecal samples were sterilely collected from MIA and control offspring at 1, 2 and 3 weeks after the start of treatment with *B. fragilis* or vehicle. Germ-free mice were treated with *B. fragilis* as described above to serve as positive controls. DNA was isolated fecal samples using the QIAamp DNA Stood Mini Kit (Qiagen). 50 ng DNA was used for qPCR with *B. fragilis*-specific primers, *5'* TGATTCCGCATGGTTTCATT *3'* and *5'* CGACCCATAGAGCCTTCATC *3'*, and universal 16S primers *5'* ACTCCTACGGGAGGCAGCAGT *3'* and *5'* ATTACCGCGGCTGCTGGC *3'* according to ref. (Odamaki et al., 2008).

Metabolome screening

Sera were collected by cardiac puncture from behaviorally validated adult mice. Samples were extracted and analyzed on GC/MS, LC/MS and LC/MS/MS platforms by Metabolon, Inc. Protein fractions were removed by serial extractions with organic

aqueous solvents, concentrated using a TurboVap system (Zymark) and vacuum dried. For LC/MS and LC/MS/MS, samples were reconstituted in acidic or basic LC-compatible solvents containing >11 injection standards and run on a Waters ACQUITY UPLC and Thermo-Finnigan LTQ mass spectrometer, with a linear ion-trap front-end and a Fourier transform ion cyclotron resonance mass spectrometer back-end. For GC/MS, samples were derivatized under dried nitrogen using bistrimethyl-silyl-trifluoroacetamide and analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. Chemical entities were identified by comparison to metabolomic library entries of purified standards. Following log transformation and imputation with minimum observed values for each compound, data were analyzed using two-way ANOVA with contrasts.

4EPS synthesis and detection

Potassium 4-ethylphenylsulfate was prepared using a modification of a procedure reported for the synthesis of aryl sulfates in refs. (Burlingham et al., 2003; Grimes, 1959). 4-ethylphenol (Sigma-Aldrich, 5.00 g, 40.9 mmol) was treated with sulfur trioxide-pyridine complex (Sigma-Aldrich, 5.92 g, 37.2 mmol) in refluxing benzene (20 ml, dried by passing through an activated alumina column). After 3.5 h, the resulting solution was cooled to room temperature, at which point the product crystallized. Isolation by filtration afforded 7.93 g of crude pyridinium 4-ethylphenylsulfate as a white crystalline solid. 1.00 g of this material was dissolved in 10 mL of 3% triethylamine in acetonitrile and filtered through a plug of silica gel (Silicycle, partical size 32-63 μm), eluting with 3% triethylamine in acetonitrile. The filtrate was then concentrated, and the resulting residue

was dissolved in 20 mL of deionized water and eluted through a column of Dowex 50WX8 ion exchange resin (K^+ form), rinsing with 20 mL of deionized water. The ion exchange process was repeated once more, and the resulting solution concentrated under vacuum to afford 618 mg (55% overall yield) of potassium 4-ethylphenylsulfate as a white powder (Supplementary Fig. 9a).

^{1}H and ^{13}C NMR spectra of authentic potassium 4-ethylphenylsulfate were recorded on a Varian Inova 500 spectrometer and are reported relative to internal DMSO-d_5 (^{1}H, δ = 2.50; ^{13}C, δ = 39.52). A high-resolution mass spectrum (HRMS) was acquired using an Agilent 6200 Series TOF with an Agilent G1978A Multimode source in mixed ionization mode (electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI)). Spectroscopic data for potassium 4-ethylphenylsulfate are as follows: ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.11 – 7.04 (m, 4H), 2.54 (q, J = 7.6 Hz, 2H), 1.15 (t, J = 7.6 Hz, 3H); ^{13}C NMR (DMSO-d_6, 126 MHz) δ 151.4, 138.3, 127.9, 120.6, 27.5, 16.0; HRMS (Multimode-ESI/APCI) calculated for $C_8H_9O_4S$ [M–K]201.0227, found 201.0225.

Authentic 4EPS and serum samples were analyzed by LC/MS using an Agilent 110 Series HPLC system equipped with a photodiode array detector and interfaced to a model G1946C single quadrupole expectospray mass spectrometer. HPLC separations were obtained at 25°C using an Agilent Zorbax XDB-C18 column (4.6 mm x 50 mm x 5 um particle size). The 4EPS ion was detected using selected ion monitoring for ions of m/z 200.9 and dwell time of 580 ms/ion, with the electrospray capillary set at 3 kV. Authentic potassium 4EPS was found to possess a retention time of 6.2 minutes when eluted in 0.05% trifluoroacetic acid and acetonitrile, using a 10-minute linear gradient

from 0-50% acetonitrile. For quantification of 4EPS in mouse sera, a dose-response curve was constructed by plotting the total ion count peak area for known concentrations of authentic potassium 4EPS against the analyte concentration (R^2=0.9998; Supplementary Fig. 9b). Mouse serum samples were diluted four-fold with acetonitrile and centrifuged at 10,000 g at 4°C for 3 minutes. 10 ul of supernatant was injected directly into the HPLC system.

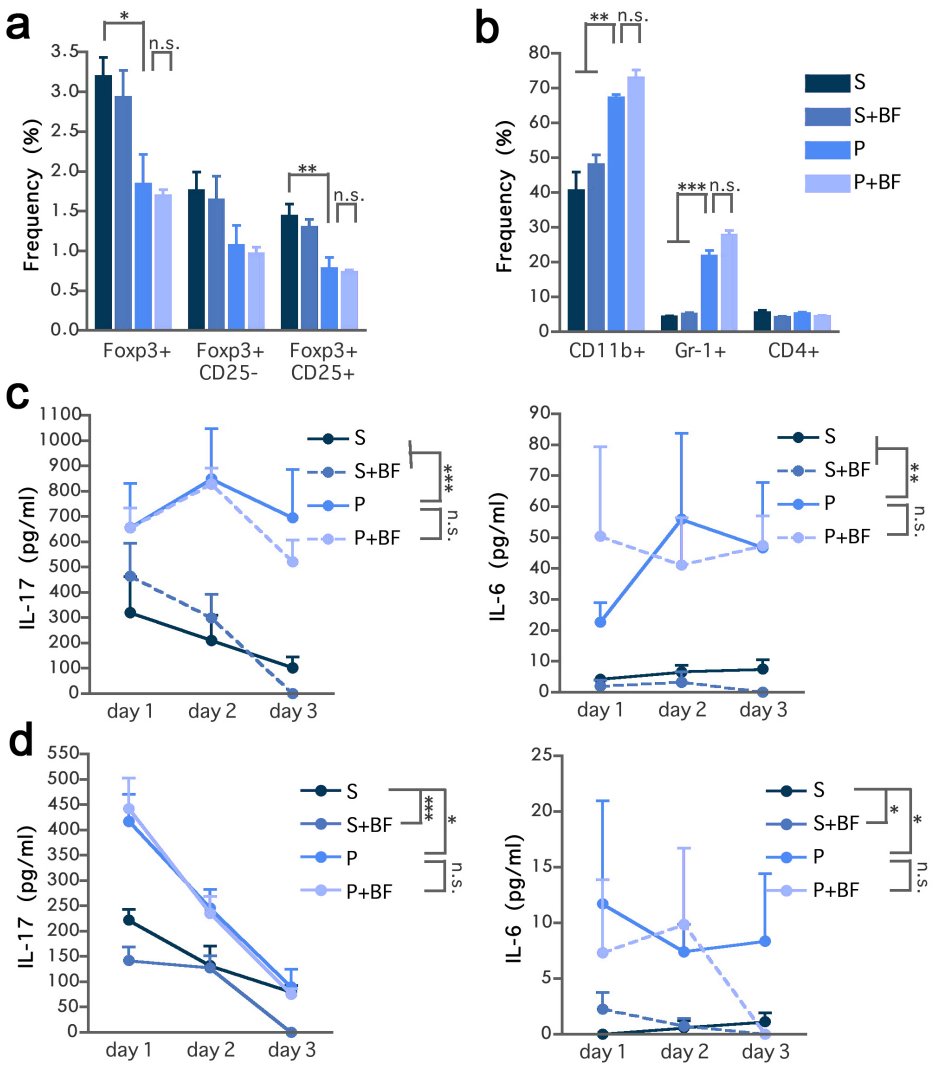
4EPS sufficiency experiments

Wildtype mice were injected i.p. with saline or 30 mg/kg 4EPS potassium salt daily from 3 to 6 weeks of age. A dose-response curve was generated by measuring serum 4EPS levels at various times after i.p. injection of 30 mg/kg 4EPS (Supplementary Fig. 9c). Mice were behaviorally tested as described above from 6 to 9 weeks of age.

Statistical Analysis

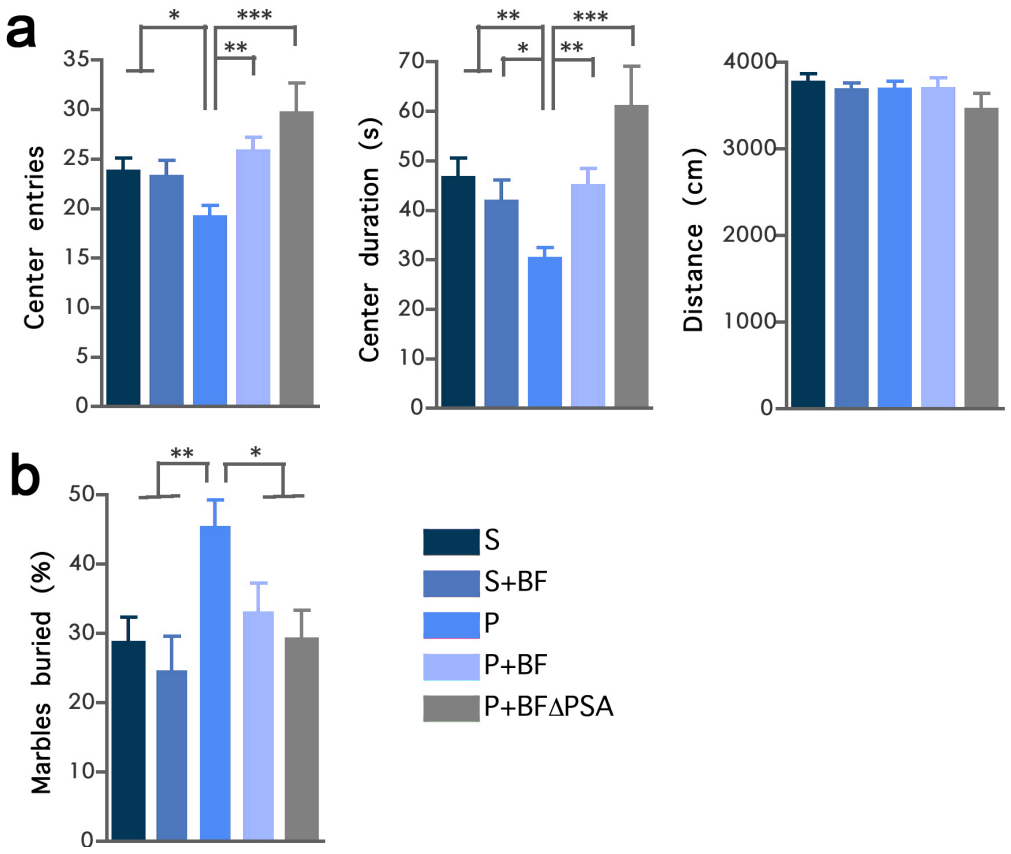
Statistical analysis was performed using Prism software (Graphpad). Data were assessed for normal distribution and plotted in the figures as mean ± SEM. Differences between two treatment groups were assessed using the Student *t* test, and differences among multiple groups were assessed using one-way ANOVA with Bonferroni post hoc test. Two-way repeated measures ANOVA with Bonferroni post hoc test was used for analysis of PPI and CD4+ T-cell stimulation data. Two-way ANOVA with contrasts was used for analysis of the metabolite data. Significant differences emerging from the above tests are indicated in the figures by **P*<0.05, ***P<0.01,* ****P*<0.001. Notable near-significant differences are indicated in the figures by φ *P*=0.05**,** # *P*<0.07 and Ψ *P*=0.08. Notable

non-significant (and non-near significant) differences are indicated in the figures by "n.s.".

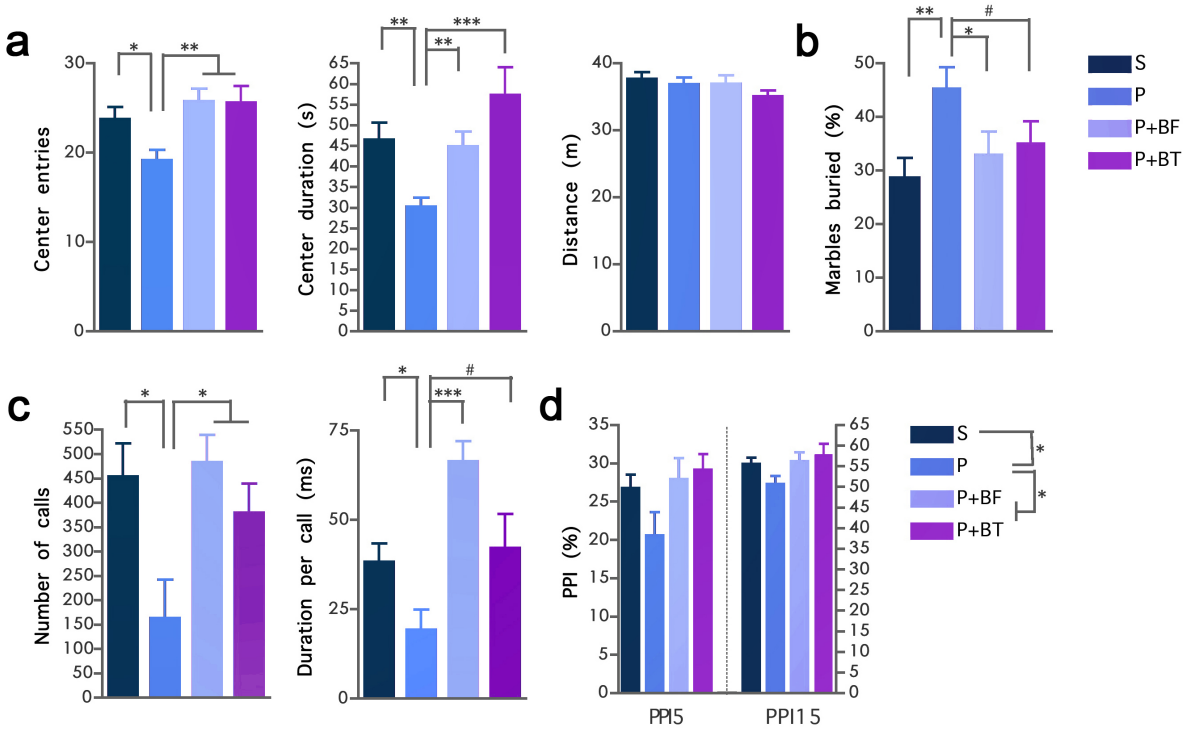


Supplementary Figure 1. *B. fragilis* treatment has no effect on systemic immune dysfunction in MIA offspring. a, After *B. fragilis* treatment, adult poly(I:C) offspring retain deficits in splenic Foxp3+ CD25+ Tregs. **b,** *B. fragilis* treatment also has no impact on the elevated levels of Gr-1+ and CD11b+ neutrophilic and monocytic cells observed in spleens of adult MIA versus control offspring. **c,** *B.* fragilis also has no effect on the hyper-responsivity of splenic CD4+ T cells observed in MIA offspring, as indicated by comparably elevated production of IL-6 and IL-17 after stimulation of CD4+ T cells derived from poly(I:C) versus *B. fragilis*-treated poly(I:C) offspring. **d,** Similarly, there is

no difference after *B. fragilis* treatment in production of IL-6 or IL-17 by CD4+ T cells derived from the mesenteric lymph nodes of MIA offspring. n = 5. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, n.s. = not significant. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF= poly(I:C)+*B. fragilis.*

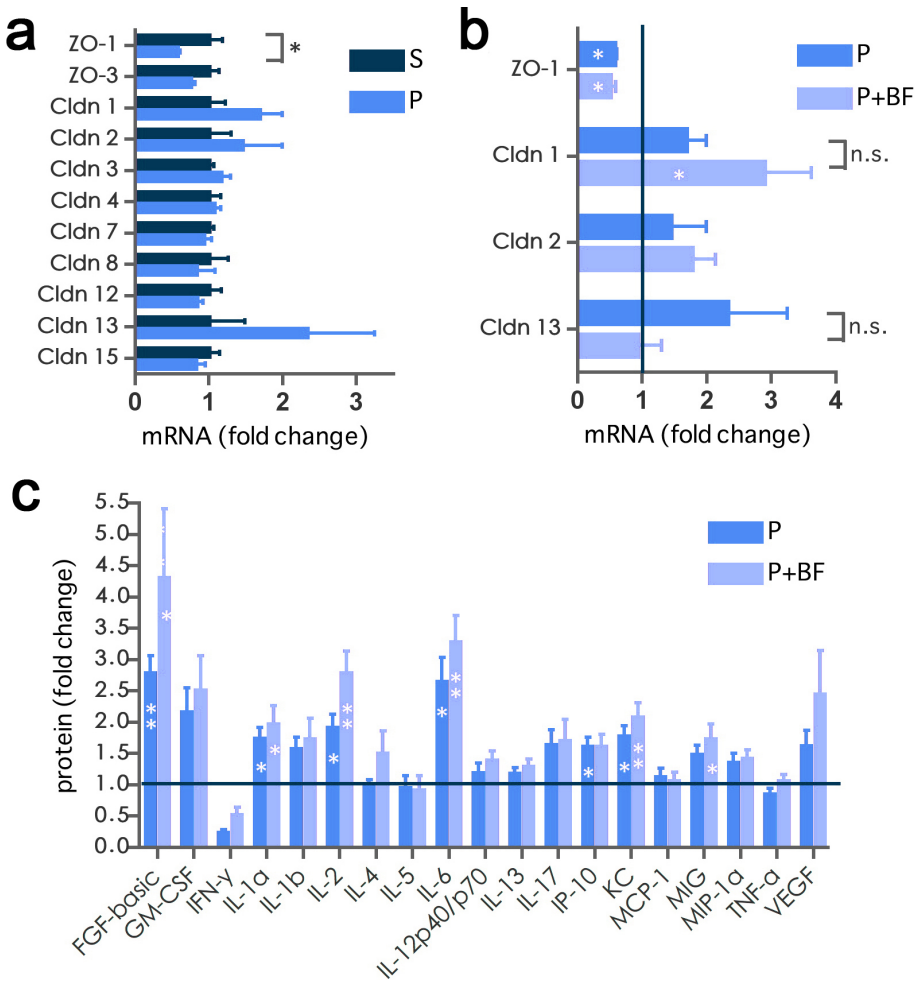


Supplementary Figure 2. *B. fragilis*-mediated amelioration of autism-related behaviors is not dependent on polysaccharide A (PSA). a, MIA offspring treated with mutant *B. fragilis* lacking PSA exhibit decreased anxiety-like behavior in the open field, with increased entries into and duration spent in the center arena comparable to that seen with wildtype *B. fragilis* treatment. There is no difference between treatment groups in general locomotion as measured by total distance traveled. **b,** Deletion of PSA has no effect on *B. fragilis*-mediated reduction in stereotyped marble burying behavior in adult MIA offspring. n= 17-75. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF= poly(I:C)+*B. fragilis*, P+BFΔPSA=poly(I:C)+*B. fragilis* with PSA deletion.



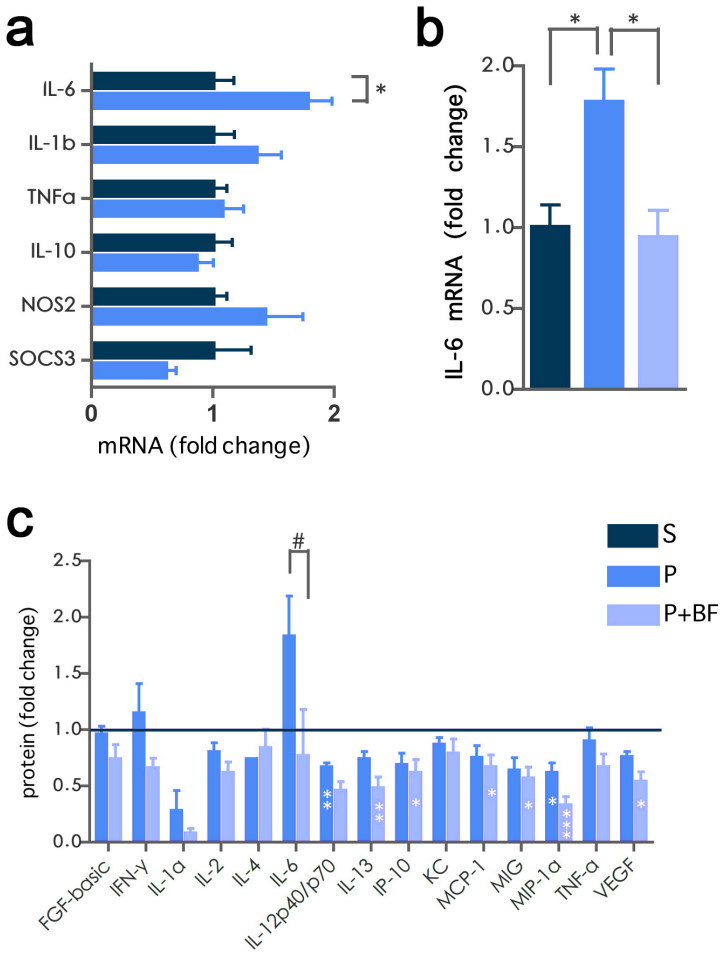
Supplementary Figure 3. Amelioration of autism-related behaviors in MIA offspring is not specific to *B. fragilis* treatment. a, MIA offspring treated with *B. thetaiotaomicron* exhibit decreased anxiety-like behavior in the open field, with increased entries into and duration spent in the center arena comparable to that seen with *B. fragilis* treatment. There is no difference between treatment groups in general locomotion as measured by total distance traveled. n= 30-75. **b,** *B. thetaiotaomicron* treatment leads to a trending decrease in stereotyped marble burying, similar to that observed with *B. fragilis* treatment. n= 30-45. **c**, Treatment of MIA offspring with *B. thetaiotaomicron* restores communicative behavior as measured by adult production of ultrasonic vocalizations, with a statistically significant increase in total number of calls produced and a trending increase in average duration per call. n= 7-10 males. **d,** *B. thetaiotaomicron* treatment significantly improves sensorimotor gating as measured by prepulse inhibition in adult

MIA offspring. n= 30-75. #$P < 0.07$, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF= poly(I:C)+*B. fragilis*, P+BT=poly(I:C)+*B. thetaiotaomicrom.*

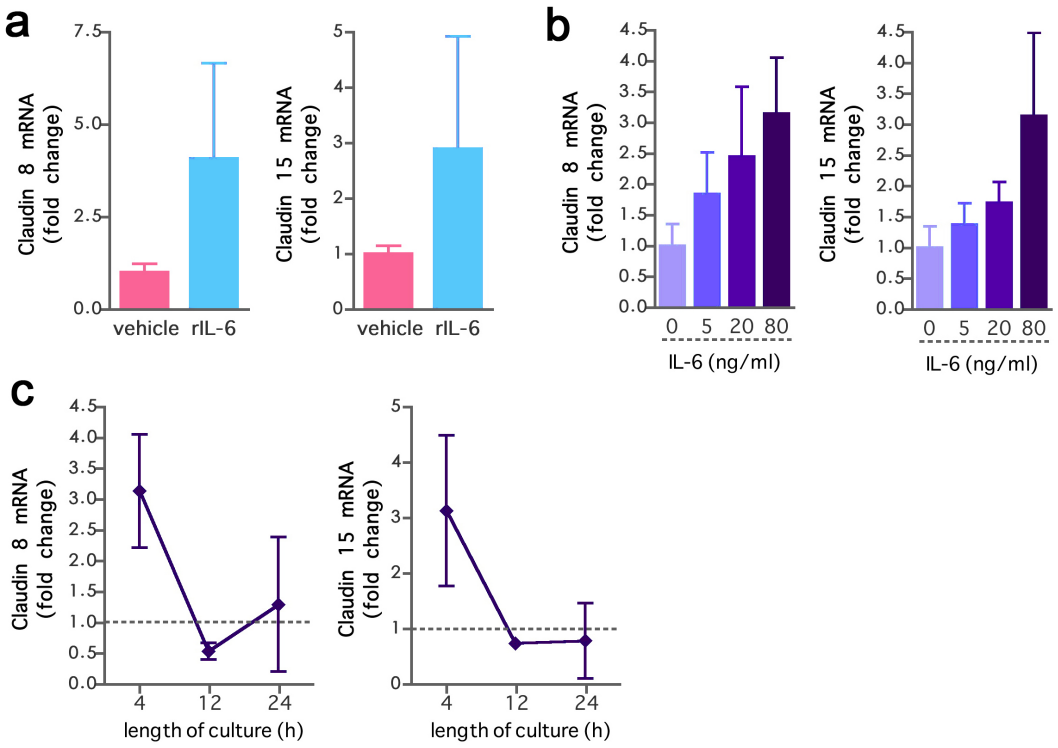


Supplementary Figure 4. *B. fragilis* treatment has little effect on tight junction expression and cytokine profiles in the small intestine. a, MIA offspring display significantly decreased expression of ZO-1 and a trending increase in expression of claudin 1 in the small intestine. n = 8 **b,** MIA offspring treated with *B. fragilis* retain their deficit in small intestine ZO-1 expression, and exhibit significantly increased small intestine claudin 1 expression. n = 8 **c,** MIA offspring display significantly elevated levels of FGF-basic, IL-1a, IL-2, IL-6, IP-10 and KC protein in the small intestine, compared to controls. *B. fragilis* treatment has no effect on these cytokine changes. Asterisks within bars denote significance compared to saline control (normalized to 1, as denoted by the black line in (b) and (c)), whereas asterisks to the right of bars denote

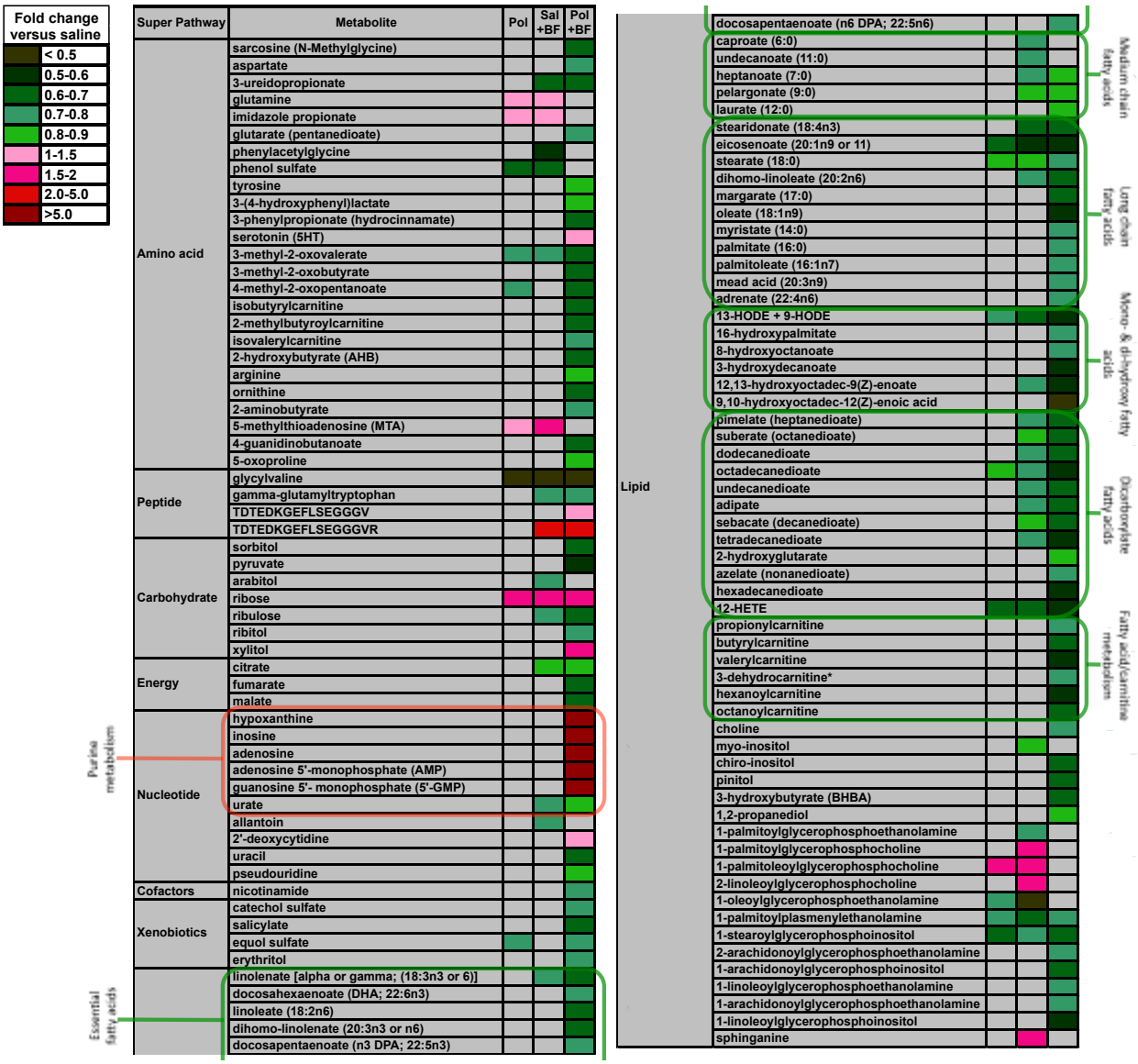
statistical significance between poly(I:C) and poly(I:C)+*B. fragilis*. n = 8-10. *$P < 0.05$, n.s. = not significant. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis*.



Supplementary Figure 5. IL-6 expression is elevated in colons from MIA offspring and is normalized by *B. fragilis* treatment. a, Compared to controls, adult MIA offspring exhibit increased expression of IL-6 mRNA in the colon, but no difference in colon IL-1b, TNFa, IL-10, NOS2 and SOCS3 expression. n = 6-21 **b,** *B. fragilis* treatment reduces levels of colon IL-6 mRNA to levels observed in saline controls. **c,** The increase in colon IL-6 is also seen at the protein level and is similarly reduced by *B. fragilis* treatment. Asterisks within bars denote significance compared to saline control (normalized to 1, as denoted by the black line in (c)). n = 10; # $P < 0.07$, *$P < 0.05$. S=saline+vehicle, P=poly(I:C)+vehicle, P+BF= poly(I:C)+*B. fragilis.*

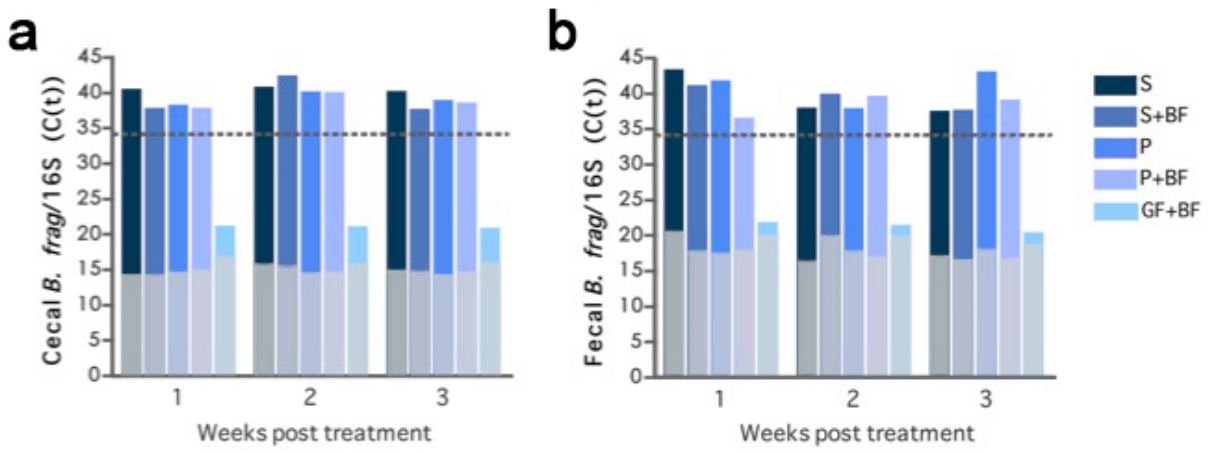


Supplementary Figure 6. IL-6 modulates colon expression of claudin 8 and 15. a, Oral gavage of recombinant mouse IL-6 leads to numerically increased claudin 8 and 15 expression at 4 hours post-gavage. **b,** IL-6 induces a dose-dependent increase in colon claudin 8 and 15 expression after 4 hours of culture. **c,** Changes in colon claudin 8 and 15 expression in response to IL-6 in culture are influenced by the duration of culture. Significant decreases in claudin 8 and 15 expression are observed after 12 hours of culture. n = 3 animals

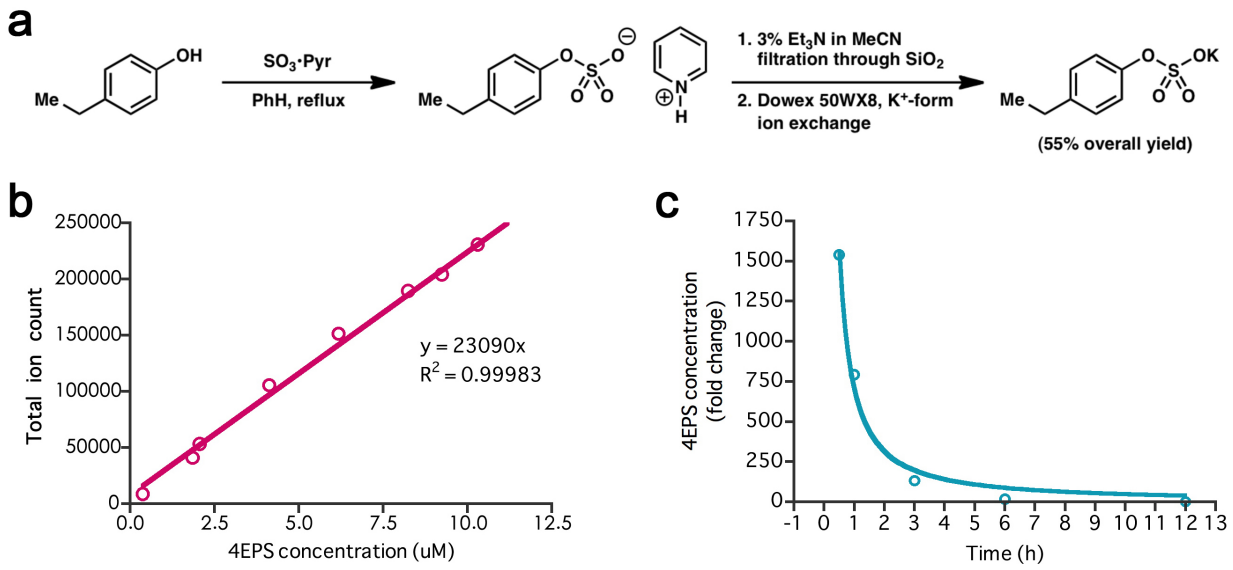


Supplementary Figure 7. *B. fragilis* treatment causes statistically significant alterations in 109 serum metabolites, with widespread changes in biochemicals relevant to fatty acid metabolism (green outline) and purine salvage pathways (red outline). n = 8. That MIA offspring display significantly decreased serum 3-hydroxybutyrate levels, a marker of liver beta-oxidation of fatty acids suggests that the widespread decrease in fatty acids is due to deficient uptake by the intestinal epithelium. Since treated offspring exhibit significantly lower levels of serum urate, an end product

of purine catabolism, the elevated levels of purine nucleosides and nucleobases likely reflect enhanced purine salvage.



Supplementary Figure 8. There is no evidence for persistent colonization of *B. fragilis* after probiotic treatment of MIA or control offspring. a, Cecal samples collected from MIA and control offspring at 1, 2, and 3 weeks after probiotic or vehicle treatment exhibit negligible qPCR signals (cycling threshold [C(t)] > 34) from *B. fragilis*-specific primers, despite showing robust signal from universal 16S primers (15<C(t)<20; translucent gray bars). As a positive control, cecal samples from germ-free mice treated with *B. fragilis* exhibit high *B. fragilis* signal (C(t)~20). **b,** Similarly, fecal samples collected from MIA and control offspring display negligible *B. fragilis* levels, despite being positive for universal 16S signal. S=saline+vehicle, S+BF= saline+*B. fragilis*, P=poly(I:C)+vehicle, P+BF=poly(I:C)+*B. fragilis*, GF+BF=germ-free+*B. fragilis*.



Supplementary Figure 9. 4-ethylphenylsulfate (4EPS) synthesis, detection and *in vivo* experiments. a, 4EPS was synthesized by treating 4-ethylphenol with sulfur trioxide-pyridine in refluxing benzene to generate the pyridinium salt followed by ion exchange over K+ resin to generate the potassium salt. **b,** A dose-response curve was generated by analyzing known concentrations of potassium 4EPS by LC/MS. Linear regression analysis results in a correlation coefficient (R^2) of 0.99983. **c,** After a single i.p. injection of 30 mg/kg potassium 4EPS, mice experience an acute elevation in serum 4EPS that peaks by 30 min post-injection and lasts over 3 hours post-injection.

Biological Super Pathway	Metabolite (fold change)
Amino acid	trans-urocanate (1.71), indolepyruvate (1.57) imidazole propionate (1.35), 5-methylthioadenosine (1.34), glutamine (1.20), phenol sulfate (0.68), 4-methyl-2-oxopentanoate (0.70), 4-methyl-2-oxopentanoate (0.70), N-acetylserine (0.73), 3-methyl-2-oxovalerate (0.75)
Peptide	glycylvaline (0.48)
Carbohydrate	ribose (1.44), 3-phosphoglycerate (0.51), phosphoenolpyruvate (0.56)
Lipid	1-palmitoleoylglycerophosphocholine (1.49), eicosenoate (0.61), stearoylglycerophosphoinositol (0.64), 12- HETE (0.69), 1-oleoylglycerophosphoethanolamine (0.70), 13-HODE+9-HODE (0.72), 1-palmitoylplasmenylethanolamine (0.73), stearate (0.88), octadecanedioate (0.83)
Cofactors and vitamins	4-ethylphenylsulfate (46.39), bilirubin (E,E) (2.68), glycolate (hydroxyacetate) (1.17), equol sulfate (0.78)

Supplementary Table 1. MIA leads to statistically significant alterations in 26 serum metabolites. Fold change compared to saline controls is indicated in parentheses. Increases are denoted by red font and decreases are denoted by green font. n = 8.

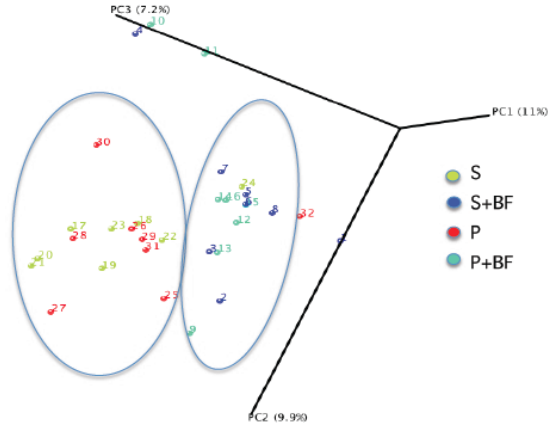
Supplementary Data 1 (Dataset online). Compiled data from LC-GC/MS-based metabolomic screening.

Supplementary Data and Discussion

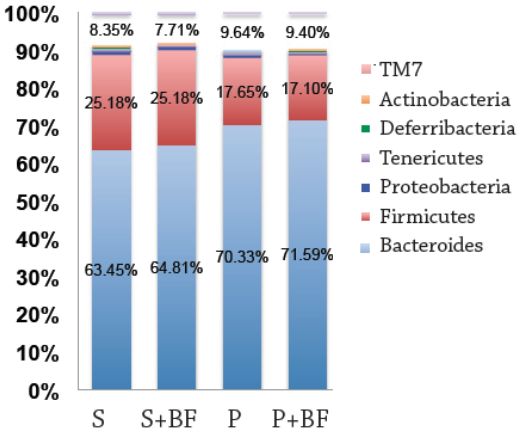
We find that *B. fragilis* treatment does not lead to persistent colonization of *B. fragilis* or to any expected effects of *B. fragilis* on immunity. However, we observe lasting effects of *B. fragilis* treatment on behavior, GI permeability and the serum metabolome, suggesting that *B. fragilis* treatment induces long term changes in the composition of the intestinal microbiome. To evaluate this, we utilized 454 pyrosequencing to examine microbial community composition from fecal samples of adult MIA and control offspring treated with or without *B. fragilis* at 10 weeks of age.

Interestingly, we find that MIA itself sufficiently leads to global alterations in the composition of the microbiota, characterized by decreased firmicutes (Lacnospiraceae), slightly increased Bacteroidetes (includes Bacteroidales, decreased Bacteroidaceae), decreased proteobacteria (Alcaligenaceae and Alphaproteobacteria) and increased Actinobacteria. Surprisingly, however, *B. fragilis* does not induce global changes in the microbiota, but rather causes considerable specific reductions in the Tenericute phylum (Eryspipelotrichaeceae) and selective loss of Betaproteobacteria in poly(I:C) offspring. Together, these data suggest that specific changes to particular species may mediate effects of *B. fragilis* treatment on host physiology and behavior.

UniFrac-Based Principle Component Analysis

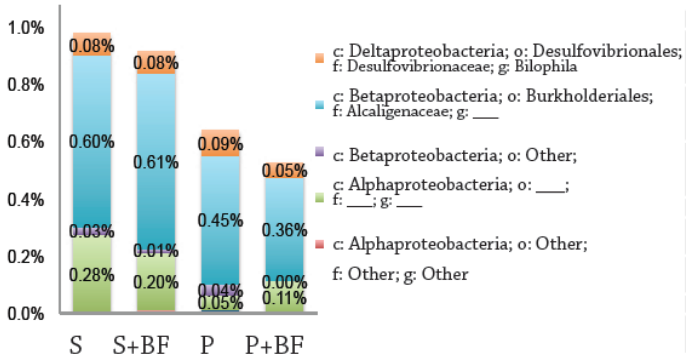


Relative Abundance of Major Phyla



Microbiome changes induced in MIA offspring by *B. fragilis* treatment:

Relative Abundance of Proteobacteria Family



Machine Learning: Unique Proteobacteria lost after *B. fragilis* treatment of MIA offspring

OTU ID #	Blast Result with Max Identity
1364	Parasutterella excrementihominis YIT 11859 ; Schlegelella thermodepolymerans strain K14;Rhodocyclus tenuis strain 2761
535	Parasutterella excrementihominis YIT 11859; Azoarcus evansii strain KB740; Azoarcus tolulyticus strain Tol-4
1492	Parasutterella excrementihominis YIT 11859 ; Thiobacter subterraneus strain C55 1 Limnobacter thiooxidans strain CS-K2
1845	Parasutterella excrementihominis YIT 11859
1279	Parasutterella excrementihominis YIT 11859 ; Azoarcus buckelii strain U120
1479	Parasutterella excrementihominis YIT 11859 ; Herbaspirillum seropedicae strain Z67 ;
1366	Parasutterella excrementihominis YIT
1261	Paraprevotella xylaniphila YIT 11841 ;Paraprevotella clara YIT 11840 strain YIT 11840

Consistent with the notion that the beneficial effects of *B. fragilis* treatment on GI physiology and behavior are mediated by long-term changes in the composition of the microbiome, we find that the ability to restore autism-related behaviors in MIA offspring by microbial treatment is not specific to *B. fragilis* itself. Rather, treatment with *B. thetaiotaomicron* also corrects for the autism-related communicative, sensorimotor, stereotyped and anxiety-like behavior in MIA offspring. We further assessed the ability to utilize other microbes to treat MIA offspring, and the data are summarized here. Overall, we see that disparate species can impart specific effects on particular behavioral

phenotypes. The mechanisms underlying these discrepancies are entirely unknown; however, it suggests that microbe treatments can be utilized to alter specific phenotypes in mice.

Behavioral Test	Poly(I:C) + B. frag	Poly(I:C) + B. frag dPSA	Poly(I:C) + B. theta	Poly(I:C) + B. vulgatus	Poly(I:C) + E. faecalis
Open field exploration					
Center duration	↑ **	↑ ***	↑ ***	↑ **	n.s.
Center entries	↑ *	↑ ***	↑ **	↑ *	n.s.
Total distance	n.s	n.s.	↓ **	n.s.	n.s.
Stereotyped marble burying					
Percent marbles buried	↓ **	↓ *	↓ p=0.07	n.s.	n.s.
Ultrasonic vocalizations					
Total # of calls	↑ *	n.s.	↑ *	↑ **	*
Total call duration	↑ p=0.06	n.s.	↑ p=0.08	↑ *	*
Average duration per call	↑ *	n.s.	↑ p=0.07	↑ *	↑ p=0.07

Finally, we begin to assess the ability to extend the beneficial effects of *B. fragilis* treatment to additional genetic and environmental models for autism. For this, we utilized the valproic acid (VPA) model which is founded on strong epidemiological links between maternal VPA exposure and the development of features of autism in the offspring. In mice, maternal i.p. injection with VPA on E12.5 has been shown to elicit autism-related behaviors in the adult offspring. We also utilize BTBR strain mice, which researchers find exhibit decreased social interaction and elevated stereotyped behaviors when compared to particular strains of mice, such as the C57Bl/6. We further utilize transgenic mice harboring a knockout allele of the CNTNAP2 gene, which encodes a synaptic protein found to be altered in human autism and to be associated with language

production. The CNTNAP2 knockout mouse was recently validated to exhibit core autism related behaviors, by researchers from the Geschwind lab at UCLA.

Results from our preliminary analysis indicate that *B. fragilis* treatment has variable effects on prepulse inhibition, open field and ultrasonic vocalization behavior across different mouse models for autism. However, in many cases we were unable to replicate reported autism-related phenotypes in the mouse models. For example, in the VPA model, no autism-related behaviors were observed in VPA offspring versus controls. Also, in the CNTNAP model, only trending changes at most were observed as compared to those reported in primary research papers using similar sample sizes. Further experiments need to be performed to optimize mouse models for autism and to gain insight into whether *B. fragilis* treatment can be translated.

Behavioral Test	Poly(I:C) + B. frag (vs Poly(I:C))	BTBR + B. frag (vs BTBR)	VPA + B. frag (vs VPA)	CNTNAP2-/- + B. frag (vs CNTNAP2-/-
Prepulse-Inhibition				
Percent PPI	↑ *	↑ **	↑ **	--
Startle	--	--	--	--
Open field exploration				
Center duration	↑ **	--	--	--
Center entries	↑ *	↑ *	--	--
Total distance	--	↑ *	↑ *	--
Stereotyped marble burying				
Percent marbles buried	↓ **	--	--	--
Self-grooming				
Time spent grooming	--	--	--	--
Social Interactions				
Sociability	--	--	--	--
Social Preference	--	↓ *	--	--
Total distance	--	↑ *	--	--
Ultrasonic vocalizations				
Total # of calls	↑ *	--	--	↑ p=0.08
Total call duration	↑ p=0.06	--	--	↑ p=0.06
Average duration per call	↑ *	--	--	--

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Appendix A

Potential impact of maternal immune activation on placental hematopoietic stem cells

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Abstract

Maternal immune activation (MIA) increases the risk for schizophrenia and symptoms of autism spectrum disorders (ASD) in humans and mice. In pregnant mice, MIA can be induced by respiratory infection or by the administration of poly(I:C), a synthetic, double-stranded RNA that triggers an anti-viral inflammatory response (Shi et al., 2003). The offspring of mothers injected with poly(I:C) at E12.5 exhibit peripheral immune dysregulation and behavioral deficits associated with schizophrenia and autism. In addition, Smith et al. revealed that the pro-inflammatory cytokine IL-6 is both necessary and sufficient to induce behavioral and transcriptional changes in the offspring (Smith et al, 2007). As IL-6 can cross the placenta and as E12.5 marks the onset of placental hematopoietic stem cell (HSC) expansion (Mikkola et al.,2006), we sought to determine whether the placenta could be a site of early peripheral immune dysregulation in an MIA mouse model. Our studies show that MIA upregulates IL-6 in the E13.5 placenta and that HSCs in the placenta at this time express TLR3. Thus, HSCs could respond to MIA induced IL-6 through cytokine receptors and/or to poly(I:C) directly through the TLR3 receptor. Real-Time PCR on CD34 dim c-Kit high HSCs isolated from E13.5 and E15.5 embryos of pregnant mothers injected with poly(I:C) reveals MIA induced trends in gene expression among saline versus poly(I:C) groups. This finding was supported by methylcellulose cultures in which the introduction of poly(I:C) to primary cells led to increased numbers of myeloid colonies. This data would suggest that poly(I:C) induced MIA may alter the fate of HSCs and progenitors in vivo. However, the observed changes in these populations may be temporary; examination of overall cell populations in the

spleens of adult mice whose pregnant mothers were injected with poly(I:C) or saline revealed no differences in abundance of general cell types between poly(I:C) and saline groups. Thus, it appears that HSCs and progenitors are resistant to transiently induced changes in gene expression.

Introduction

Schizophrenia and Autism Spectrum Disorders (ASD) include severe behavioral and developmental deficits (DSMIV TR). Schizophrenia affects roughly 1.0% of the population over the age of eighteen while the diagnoses of ASD cases are rising at an alarming rate. Also of concern are the high annual costs of these disorders. There is therefore a great need to further our understanding of the development of schizophrenia and ASD in order to provide better treatment and/or prevention of these disorders.

While there are a number of factors that have been shown to contribute to the pathology of ASD and schizophrenia, a significant environmental influence is the infection of mothers during pregnancy (Togichi et al., 2004). In humans, infection during the second trimester increases the risk for schizophrenia and symptoms of ASD in the offspring. Epidemiological studies show that there is increased incidence of schizophrenia following outbreaks of influenza with the second trimester being especially significant (Brown and Susser, 2002; Brown et. al, 2004; Brown, 2006,). In mice, poly(I:C) induced MIA is used as a model of influenza infection (Smith et al, 2006; Patterson, 2009; Urs et al, 2008, Shi et al, 2003). A single intraperitoneal injection of poly(I:C) on embryonic day E12.5 induces behavioral deficits in the offspring that are associated with schizophrenia and ASD. Shortly after MIA, cytokine levels in the placenta, maternal serum, amniotic fluid and fetal brain are altered. The cytokine IL-6 is induced in maternal serum by MIA and is critical for mediating the behavioral and transcriptional changes observed in these offspring (Smith et al., 2007).

In addition to behavioral deficits, the adult offspring display dysregulated peripheral immunity. Elevated levels of IL-17 and IL-6 are produced by CD4+ T cells

from lymph nodes and spleens of adult MIA offspring. Similar abnormalities are observed in CD4+ T cells isolated from spleens of young and newborn MIA offspring. Likewise, T cell dysregulation has been reported in humans with autism. Mostafa et al. showed that children with autism have lower levels of CD4+CD25+ T cells with a positive correlation between the severity of symptoms and the deficiency of this cell population (Mostafa et al., 2010). In addition, it has been shown that children with ASD have altered levels of naïve CD8+ cells (CCR7+ CD45Ra+), CD8+ effector memory (EM) cells (CCR7-/CD45RA-), and CD4+ terminally differentiated (TD) cells (CCR7- /CD45RA+) (Sarsella et al., 2009).

While the above studies shed further light on the complexities of ASD and schizophrenia, there is much information that remains to be discovered about the etiology of these disorders. We have investigated the effects of MIA on the development of HSCs in the placenta and fetal liver as well as possible subsequent alterations in myeloid and lymphoid populations. The development of HSCs is of particular interest as several reports demonstrate that the time of poly(I:C) injection, E12.5, marks the onset of placental hematopoietic stem cell (HSC) expansion (Mikkola et al., 2005) as well as HSC seeding of the fetal liver (Mikkola et al., 2006). In addition, embryonic HSCs express cytokine and toll-like receptors and may therefore respond to poly(I:C) directly or to poly(I:C)-induced proinflammatory cytokines (Dorner et al., 2009; McKinstry et al., 1997; Nagai et al., 2006). Thus, MIA may initiate the dysregulation of peripheral immunity in the offspring by affecting the development of the immune system at key sites during embryogenesis.

Pregnant mice were injected with poly(I:C) at E12.5, and IL-6 levels were evaluated at 24 and 72 hours post-injection in the placentas and fetal livers. HSCs were then isolated from placentas and fetal livers of E13.5 and E15.5 embryos. Real-Time PCR of these cells revealed that HSCs express TLR3 and could therefore be responding directly to poly(I:C). To examine aberrations in lineage potential, expression of transcription factors for myeloid and lymphoid progenitors were evaluated in the isolated HSCs. This data shows trending differences in gene expression of GATA-1 and IL-7Rα placental and fetal liver HSCs from poly(:C) versus saline injected mothers. Methylcellulose assays were also used to examine effects of poly(I:C) in vitro on lineage potential of fetal liver cells. To further evaluate alterations of lineage potential in vivo, abundance of adult MIA offspring spleen cell populations was evaluated for differences in saline and poly(I:C) groups.

Results

MIA elevates IL-6 levels in the Placenta and Fetal Liver

As IL-6 has was previously shown to be crucial in mediating the effects of MIA, we examined overall levels of IL-6 in the tissues and supernatant of E13.5 and E15.5 fetal livers and placentas of poly(I:C) or saline injected pregnant mothers. IL-6 is significantly upregulated in the placentas of poly(I:C) embryos at E13.5. The fetal liver shows a similar trend, but the results are not significant. By E15.5 these changes are no longer apparent in either the placenta or the fetal liver (figure 1).

MIA has no Effect on IL-17 levels in the E13.5 and E15.5 Placenta or Fetal Liver

We were also interested in examining IL-17 levels in the placenta and the fetal liver as IL-17 has been implicated in the dysregulated peripheral immunity of MIA in mice. ELISA was used to assess IL-17 levels in whole placenta and fetal liver tissues, and expression was normalized to total protein of the tissues. No significant differences were observed in IL-17 levels at E13.5 or E15.5 in either the placenta or the fetal liver.

HSCs express toll-like receptor 3

HSCs have various cytokine receptors, including the receptor for IL-6 (McKinstry et al, 1997). Thus, HSCs can respond directly to IL-6 through the IL-6 receptor. In addition, HSCs have various toll-like receptors (Nagai et al., 2006). As poly(I:C) signals through toll-like receptor 3, we were interested in whether or not placental CD34 dim c-Kit high HSCs have toll-like receptor 3. We used the markers CD34 and c-Kit as opposed to SLAM family markers, because Daley et al showed that while the placenta contains a large number of HSCs at E12.5, these cells most likely lack the SLAM family markers CD48 and CD150 as evidenced by repopulation assays (Daley et al., 2009). After cell sorting Real-Time PCR was used to evaluate TLR3 expression. TLR3 expression was observed in both fetal liver and placental HSCs. Although not significant, the TLR3 expression is lower in HSCs embryos from poly(I:C) versus saline injected mothers. This difference suggests that poly(I:C) may be inducing a downregulation of TLR3 in HSCs, perhaps through a negative feedback mechanism (figure 3). In order to visualize TLR3 in the placenta, we performed IHC on E13.5 saline tissues (figure 4).

MIA has no Effect on the Abundance of HSCs in the Placenta or Fetal Liver

As IL-6 is upregulated at E13.5 and as HSCs express TLR3, we examined potential effects of MIA on abundance of HSCs in the placenta and fetal liver 24 hours post-poly(I:C) injection. Previous studies have shown that IL-6 and poly(I:C) alters the differentiation and/or proliferative capacities of HSCs (Essers et al., 2009; Suzuki et al., 1989). HSCs were isolated from the placenta and fetal liver using fluorescence activated cell sorting (figure 5). HSCs were CD34 dim c-Kit high and progenitors were single positive for either CD34 or c-Kit. Percent totals of cell populations reveals no differences in saline versus poly(I:C) samples (figure 5). IHC for the nascent HSC and progenitor marker CD41 also reveals no differences in saline versus poly(I:C) tissues (figure 6).

Poly(I:C) Induces Myeloid Lineage Potential In Vitro but not In Vivo

To evaluate potential effects of poly(I:C) on differentiation potential, colony forming methylcellulose assays for myeloid potential were done in the presence or absence of increasing concentrations of poly(I:C). E12.5 fetal liver were roughly minced and left overnight at 37°C with the indicated concentrations of poly(I:C) or vehicle. Single cell suspensions were prepared on E13.5, and cells were plated on methylcellulose (Stem Cell Technologies) supplemented TPO (5ng/mL). Myeloid colonies were scored on day 5 according to morphological differences. The results indicate that in vitro, poly(I:C) enhances the lineage potential of fetal liver cells (figure 7A). These results then led us to investigate the effects of poly(I:C) in vitro over a longer time. For this experiment we used the same procedure, but poly(I:C) was only added at the concentration of 25ug/mL, and colonies were scored at day 12. The effect of poly(I:C) on total colony count was no longer apparent by day 12 (figure 7B). We then repeated a

similar experiment, but poly(I:C) was administered in vivo by i.p. injection of E12.5 pregnant mothers rather than being added to the culture medium. On E13.5, fetal liver cells were made into single cell suspension and plated on myeloid promoting methylcellulose. However, no differences in total colony counts were observed at days 5 or 12 (figure 8).

Alterations in Gene Expression of MIA HSCs and Progenitors

The in vitro assays with poly(I:C), the upregulation of IL-6, and the alterations in TLR3 expression led us to further investigate if MIA causes changes in HSC lineage potential in vivo. Pregnant mice were injected with 20mg/kg poly(I:C) or vehicle at E12.5. On E13.5 placental and fetal liver HSCs and progenitors were isolated by FACS as previously described. Real-Time PCR was used to evaluate gene expression levels of the common myeloid marker GATA-1 and the common lymphoid marker IL-7Rα. HSCs are CD34 dim c-Kit high and progenitors are either CD34+ or c-Kit+. We also sorted the CD34 high c-Kit high population. There are some trends that indicate MIA induced alterations in gene expression. However, these changes are not significant. The figures below show if expression of GATA or IL-7Rα is higher in the saline or poly(I:C) groups. N/A indicates that no expression level was detected. Consistencies among time points are highlighted in red.

Spleen Cell Populations in Adult MIA Offspring

The observed changes in GATA-1 and IL-7Rα is not significant as there is high variation among the samples. In some cases, the fold induction of the relative expression

of poly(I:C) over saline for a sample is 20 fold and in other instances there is no difference. Thus, in order to determine if these gene expression changes were leading to long-term alterations in cell populations, we evaluated overall cell populations in the adult offspring of mothers that were injected with either saline or poly(I:C) at E12.5. Again, these results are not significant.

Discussion

Poly(I:C) induced MIA is a useful model for studying symptoms of ASD and schizophrenia in mice. However, the mechanisms by which these disorders develop remain unclear. Previous studies have linked poly(I:C) induced MIA to behavioral deficits associated with ASD and schizophrenia and to dysregulated peripheral immunity. As the cytokine IL-6 is crucial to poly(I:C) induced MIA and as IL-6 can cross the placenta, we hypothesized that MIA could aberrantly affect the development of HSCs during embryogenesis. ELISAs confirmed the transient upregulation of IL-6 following MIA in the placenta at E13.5. In addition, we were able to observe that TLR3 is expressed in placenta tissue, and more specifically in HSCs. Thus, it is possible that placental HSCs can respond not only to the effects of MIA induced IL-6 but also to the introduction of poly(I:C) via TLR3. Furthermore, methylcellulose assays in which poly(I:C) was introduced into the media for a 24 hour period prior to plating revealed that poly(I:C) can influence the lineage potential of HSCs for up to 5 days in vitro. We did not observe similar results in the "in vivo" methylcellulose experiments. However, as there are many inconsistencies between cell culture experiments and in vivo work, we decided to investigate potential differences in lineage potential of HSCs and progenitors isolated

from E13.5 and E15.5 placentas and fetal livers. While alterations in gene expression for the myeloid marker GATA-1 and the lymphoid marker IL-7Rα were revealed, these changes were transient and insignificant. Likewise, no differences in spleen cell populations of adult MIA offspring were observed. Thus, it appears that while MIA is capable of inducing short-term changes in HSCs and progenitors, the significance of these changes is highly variable among samples. It may also be that HSCs harbor resistance to transient changes as they migrate between niches, as suggested by Kiel et al., 2005. Their study revealed that unlike stem cells of the nervous system, HSCs do not retain phenotypic, functional, or gene expression differences as they migrate between various niches (Kiel et al, 2005). Mikkola et al. showed that while the placenta supports HSCs, it does not promote their differentiation. However, the fetal liver does promote the differentiation of HSCs. Thus, a more significant injection time might be at E15.5, when the fetal liver HSC population reaches its peak (Mikkola et al., 2005). Another possibility is that while overall cell type is not affected, cell functioning may remain altered. Such deficiencies may not become apparent without additional insult, and further investigation would be needed to fully explore these possibilities.

Methods

Mice

Female C57BL/6 mice were maintained at the in-house breeding facility at Caltech University. All mice were weighed and were randomly assigned to either poly(I:C) or control groups. At E12.5, mice were injected i.p. with 20mg/kg poly(I:C) (potassium salt; Sigma, St. Louis, MO) dissolved in saline or with saline alone. Mice were sacrificed by i.p. injection with 200uL of sodium pentobarbital (Nembutal).

ELISAs for IL-6 and IL-17

ELISAs were performed on fetal liver and placenta tissues and serum from E13.5 and E15.5 embryos whose mothers were injected with either saline or poly(I:C) at E12.5. Serum refers to supernatant collected during the HSC isolation described below. Cytokine levels of serum samples were normalized to cell count of the suspension. Tissues were frozen in liquid nitrogen, and cytokine levels were normalized to total protein of the tissues with BCA assay.

Immunohistochemistry

Whole Placentas from E13.5 embryos were fixed in 4.0% PFA for 2 hours at 4°C. Tissues were then washed with PBS and left overnight at 4°C covered in 30.0% sucrose. Sucrose was removed and OCT added, and tissues were left for 1 hour at 4°C to equilibrate. Tissues were removed and placed into plastic molds, covered with OCT, frozen in liquid nitrogen, and stored at -80°C until sectioning.

For TLR3 staining, tissues were cut into 20um sections. Antigen retrieval and endogenous peroxidase blocking was performed prior to blocking in normal goat serum. Primary antibody was then added at a final concentration of 1ug/mL diluted in blocking buffer, and tissues were left overnight at 4°C in a humidified chamber. The following day the slides were washed, and a 1/400 dilution of the secondary antibody goat anti-rabbit IgG was added. Slides were incubated with the primary antibody for 1 hour at room temperature. The slides were washed and the substrate was added (ABC solution, CAT) and left to develop for 30 minutes at room temperature.

For HSC staining, we followed the procedure described in *Current Protocols* (Mikkola et al., 2008).

Serum Collection and Isolation of Fetal Liver HSCs

Mice were injected with either poly(I:C) or saline on E12.5 as previously described. At E13.5 and E15.5 mice were sacrificed with 200uL Nembutal. Conceptuses were removed and washed in HBSS with Calcium and Magnesium and 5%FBS and DNase (Sigma 9003-98-9). Fetal livers were then removed from the embryos and placed in falcons containing the above media. For the E13.5 time point, 6 saline livers from one mouse were placed into a tube with 6 livers from another mouse for a total of 12 tissues per tube. For the E15.5 time point, 5 saline livers from one mouse were placed into a tube with 5 livers from another mouse for a total of 10 tissues per tube. Only 10 tissues total were used per E15.5 group because of the expected higher HSC yields at this time (Mikkola et al., 2005). Single cell suspensions were prepared by filtering the tissues through sterile mesh filters into new falcons. All falcons were centrifuged 10’at 1200rpm at 4°C.

Supernatant was removed and stored at -80°C for ELISAs. RBC lysis was then performed on the cells, and the remaining cells were washed and filtered. Cell counts were done using 0.25% trypan blue. Cells were then incubated in FC Block and dead and lineage positive cells were depleted using Miltenyi Biotec cell columns and reagents. The remaining lineage negative cells were again incubated with FC block and stained with various antibodies diluted in PBS with Magnesium and Calcium at 50uL final volume. Cells were filtered immediately before sorting, and 20uL 7ADD was added to the cell solution. Cells were gated into 4 groups: $CD34^{high}$, $Ckit^{high}$, $CD34^{dim}$ $Ckit^{high}$, and $CD34^{high}$ $Ckit^{high}$. Sorted cells were stored in RNase/DNase free eppendorfs with 0.5mL RNA Cell Protect at -80°C.

Serum Collection and Isolation Placental HSCs

Mice were injected with either poly(I:C) or saline on E12.5 as previously described. At E13.5 or E15.5 mice were sacrificed with 200uL Nembutal. Conceptuses were removed and washed in HBSS with Calcium and Magnesium and 5%FBS and DNase (Sigma 9003-98-9). Placentas were separated and the deciduas and spongiotrophoblasts were removed. Placentas were then placed in falcons containing the above media: 6 saline/poly(I:C) placentas from one mouse were placed into a tube with 6 saline/poly(I:C) placentas from another mouse for a total of 12 tissues per tube. All falcons were centrifuged 10'at 300xg at 4C, and the supernatant removed. Cells were then resuspended in 5.0mL of a 0.1%(w/v) collagenase solution diluted in PBS with 10%FBS and 1%P/S. Mechanical dissociations were performed on the cells using a 16G needle followed by an 18G needle. The cell suspensions were then incubated 30' at 37°C. Following the

incubation, the cells were centrifuged 10'at 1200rpm and resuspended in 2.0mL HBSS. The cells were then mechanically dissociated using a 20G needle, 2.0mL 0.25% trypsin was added, and the cells were incubated 10' at 37°C. HBSS with 5%FBS was added to the cell solutions to deactivate the trypsin, and cells were centrifuged 10'at 1200rpm and resuspended in HBSS with 5% FBS. The cells were then dissociated further with 22G and 25G needles, centrifuged, and the supernatant was stored at -80°C for ELISAs. RBC lysis, cell counts, and dead cell removal was then performed as described above. The lineage depletion was also done as previously described with the exception of an additional incubation with a cytokeratin Ab to deplete trophoblasts. Cell staining and sorting was done as described above.

Antibodies and Reagents for FACS

CD34/Alexa Fluor 647 1/75 dilution (eBioscience #51-0341), Terr119/PerCPCy5.5 1/250 (Biolegend #116227), SA/PerCPCy5.5 1/100 (Biolegend #116227), CkitACK45/PE 1/75 for livers (BD Pharmingen #553869 clone ACK45,), Ckit2B8/PE 1/50 for placentas (BD Biosciences clone 2B8 #553355, BD Biosciences), CD41/FITC 1/150 (CAT), 7AAD (BD Pharmingen #559925), Cytokeratin 18/Biotin (abcam #ab27553), CD16/32 (eBioscience #14-0161), Lineage Cell Depletion Kit (Miltenyi Biotec #130-090-858), Dead Cell Removal Kit (Miltenyi Biotec 130-090-101)

RTPCR

Total RNA was isolated with the RNeasy QIAGEN kit, and cDNA was synthesized with the iscript cDNA synthesis kit (Biorad). cDNA was purified with PCR

purification kit (Qiagen). Real-Time PCR was done using SYBR green with ROX (Roche) on the ABI Prism 4300.

Primer Sequences:

GATA-1 sense, TCCTCTGCATCAACAAGCCCA

GATA-1 anti-sense GTTGAGCAGTGGATACACCTG

β-actin sense AGAGGGAAATCGTGCGTGAC

β-actin anti-sense CAATAGTGATGACCTGGCCGT

IL-7Rα sense GGATGGAGACCTAGAAGATG

Il-7Rα anti-sense GAGTTAGGCATTTCACTCGT

TLR3 sense ACCTTTGTCTTCTGCACGAACCT

TLR3 anti-sense AGTTCTTCACTTCGCAACGCA

GEMM Methylcellulose Assays

GEMM methylcellulose assays refer to lineage potential assays for colony forming units of granulocytes, erythrocytes, monocytes, and macrophages. Two types of methylcellulose assays were performed. The first assay will be referred to as the in vitro assay. In this assay, fetal liver tissues were isolated from E12.5 C57BL/6 mice and roughly minced. Equal numbers of tissues from 2 mice were combined on a 6cm petri and left overnight at 37°C with IMDM supplemented with 2%FBS and 1%P/S. Half of the dishes also had 25ug/mL poly(I:C) added to the media while other dishes half of the dishes had extra media. After 24 hours, tissues were mechanically dissociated to make single cell suspensions. Cells were plated on 35mm petris (Stem Cell Technologies #27110) with methylcellulose (Methocult 3434, Stem Cell Technologies) supplemented

with 5ng/mL TPO (recombinant mouse TPO #488-TO, R&D Systems) at the density of 2x10^4 cells per plate. Colonies were scored at days 5 and 12.

The second type of assay will be referred to as the in vivo assay. In this assay, pregnant C57BL/6 mice were injected at E12.5 with 20mg/kg saline or poly(I:C). Mice were sacrificed at E13.5, and single cell suspensions were made from fetal livers. Cells were then plated as described above and scored at days 5 and 12.

Flow Cytometry

Flow Cytometry was performed on cells isolated from spleens of C57BL/6 adult mice whose pregnant mothers were injected at E12.5 with saline or with poly(I:C). Spleens were removed, and single cell suspensions prepared by mashing the spleens through 0.2um cell filters and washing with CLB buffer. RBC lysis was performed, and cells were fixed and stained at a final count of 2x10^6 cells per trial.

Antibodies for Flow Cytometry

IL-17A/APC (eBioscience #17-7177-81), CD62L/APC (Biolegend # 104411), CD44/PE (Biolegend #103007), CD4/FITC (Biolegend # 100405), CD4/PE (Biolegend # 100407), CD8/FITC (Biolegend # 100705), Gr-1/APC (Biolegend # 108411), CD11b/APC (Biolegend # 101211), B220/FITC (Biolegend # 103205), NK1.1/PE (Biolegend # 108707)

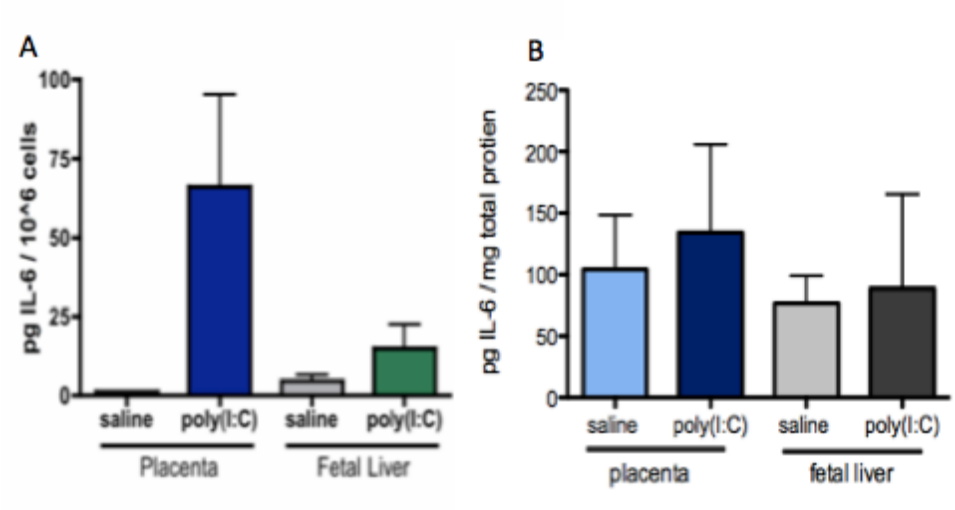

Figure 1. MIA Induced IL-6 in the Placenta and Fetal Liver: (A) IL-6 levels in the E13.5 placental and fetal liver supernatants collected from HSC isolation preparations. IL-6 is significantly upregulated in the placenta, but the upregulation in the fetal liver is not significant. (B) E15.5 IL-6 levels in placenta and fetal liver tissues. There are no longer any differences in IL-6 levels between saline and poly(I:C) groups.

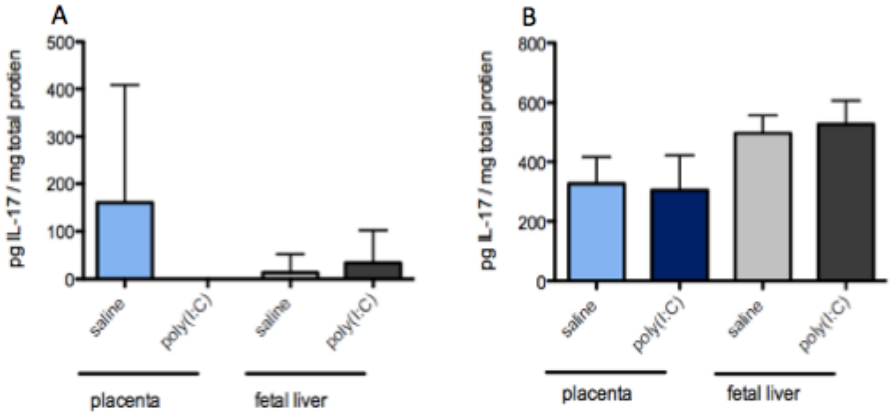

Figure 2. MIA has no effect on Il-17 levels in the placenta or fetal liver

(A) IL-17 levels in the placenta and fetal liver at E13.5 (B) IL-17 levels in the placenta and fetal liver at E15.5

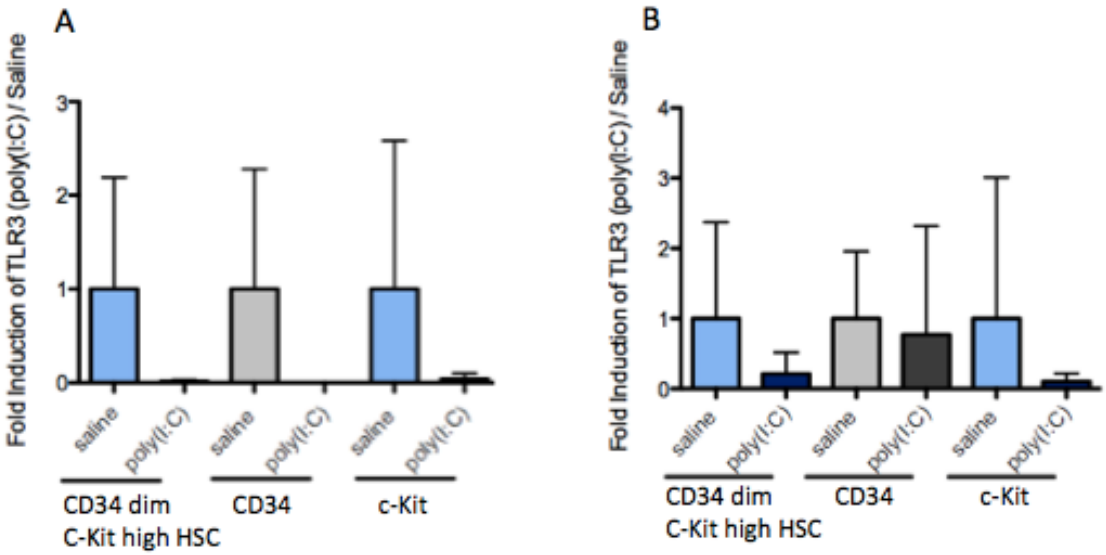

Figure 3. TLR3 expression in E13.5 Placenta and Fetal Liver HSCs (A). Fold induction of TLR (poly(I:C)/saline) in E13.5 placental HSCs (B) Fold induction of TLR (poly(I:C)/saline) in E13.5 fetal liver HSCs

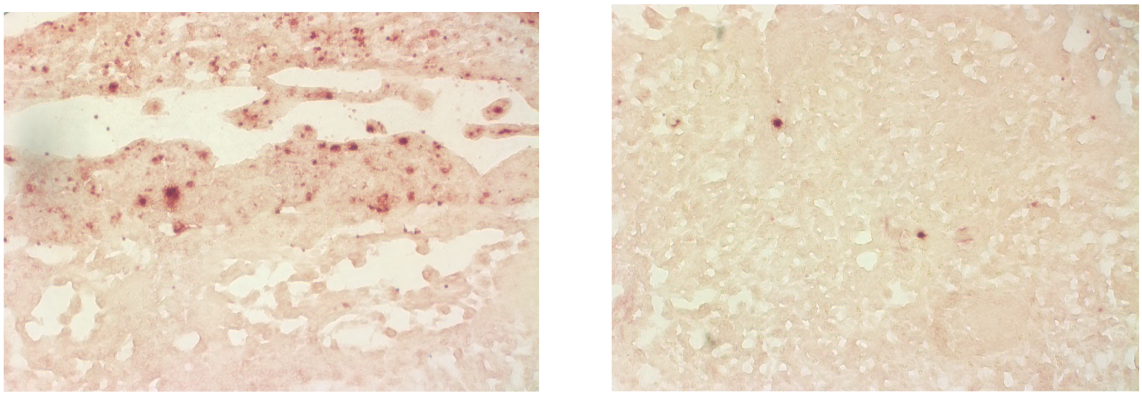

Figure 4. TLR3 staining in E13.5 Placenta, 10X objective (A). TLR3 in the decidua (B) TLR3 in the labyrinth

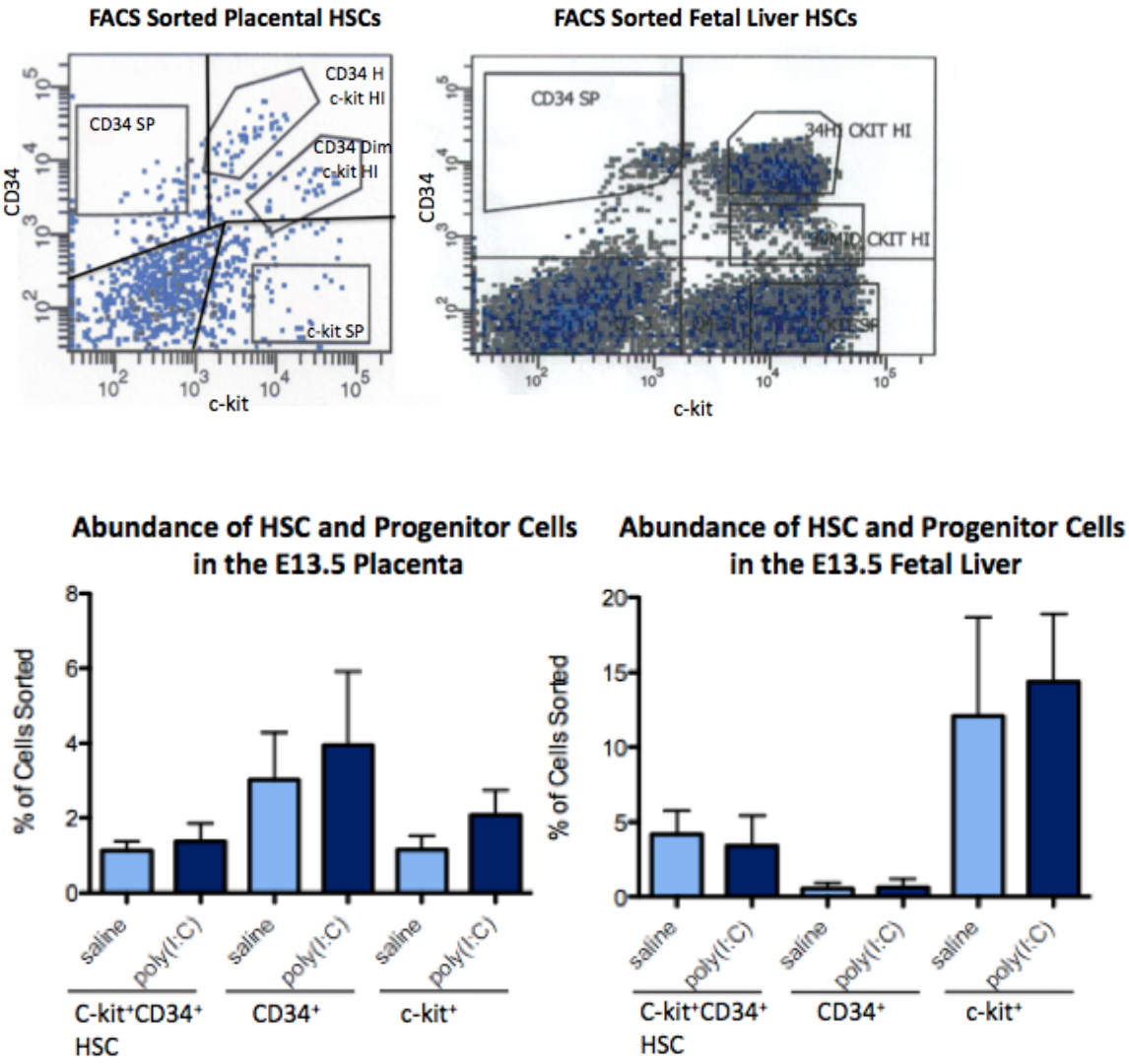

Figure 5. (Top) Representative FACS plots of HSCs (CD34 dim c-Kit High) and progenitors (CD34+ or c-Kit+) in the placenta and fetal liver

(Bottom) Percent total HSC and progenitor placenta and fetal liver cells sorted at E13.5. Percent of cells sorted refers to the percent of a specific cell type within the parent population.

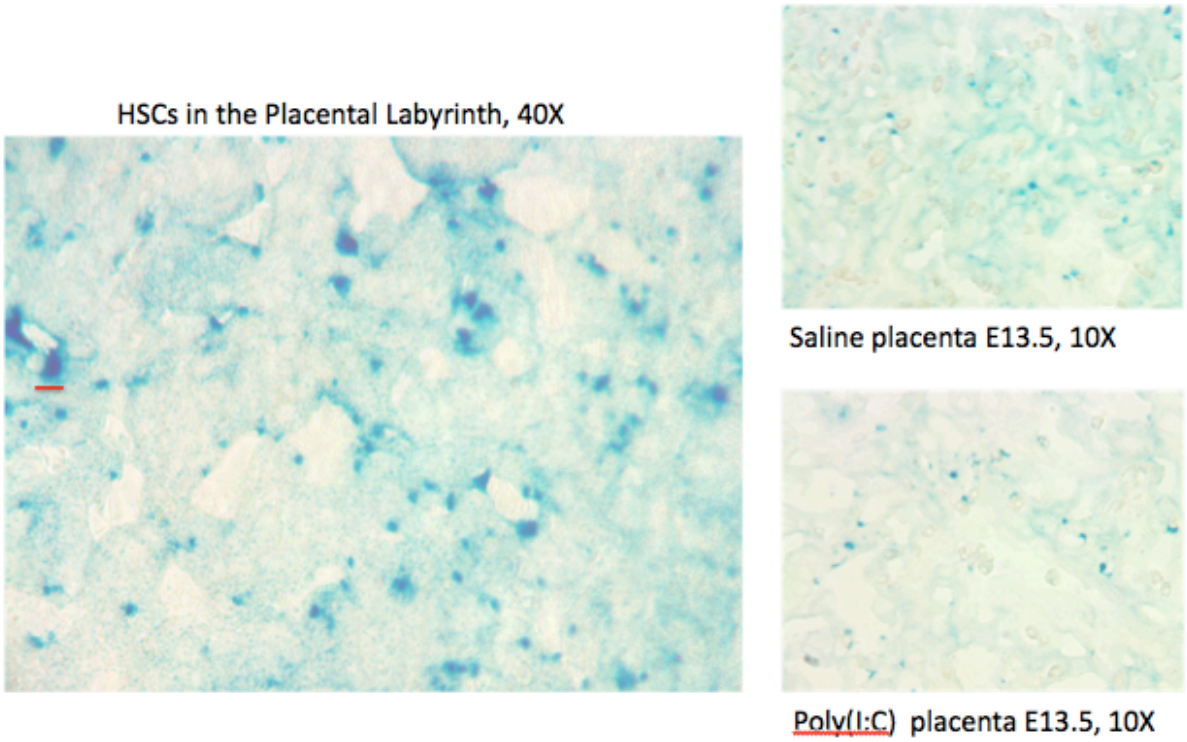

Figure 6. (Left) Red dash indicates a CD41+ HSC in the placental labyrinth at E13.5. HSCs range in size from about 8-12um, and the dash represents 10um. CD41 also stains platelets, but these are relatively smaller in size than HSCs. (Right) E13.5 CD41+ HSCs in the placental labyrinth of saline and poly(I:C) tissues. There is no difference in the abundance of HSCs in these tissues as indicated by IHC.

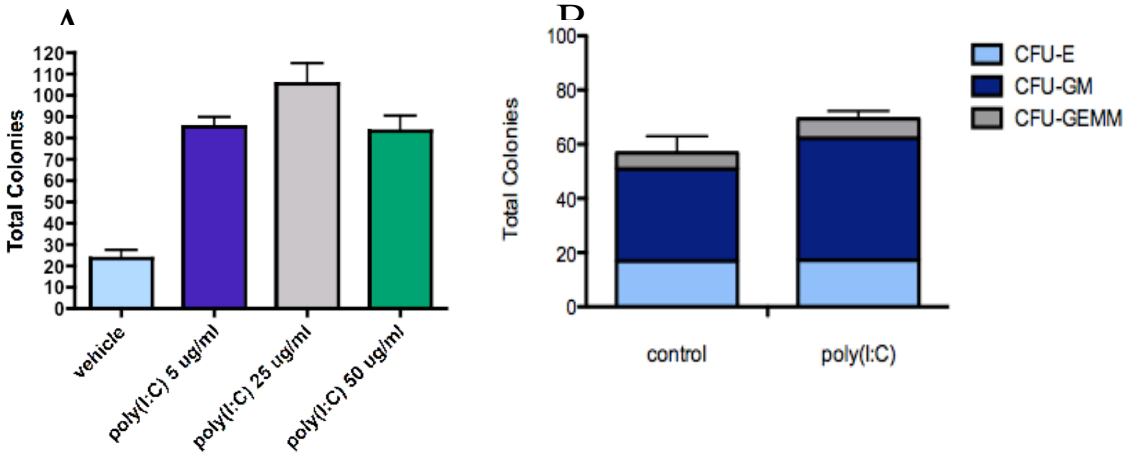

Figure 7. (A) Total Myeloid Colonies of Fetal Liver Primary Cells In Vitro Day 5. (B) Total Myeloid Colonies of Fetal Liver Primary Cells In Vitro Day 12

CFU-E (Erythroid), CFU-GM (granulocyte, macrophage), CFU-GEMM (granulocyte, erythroid, monocyte, macrophage)

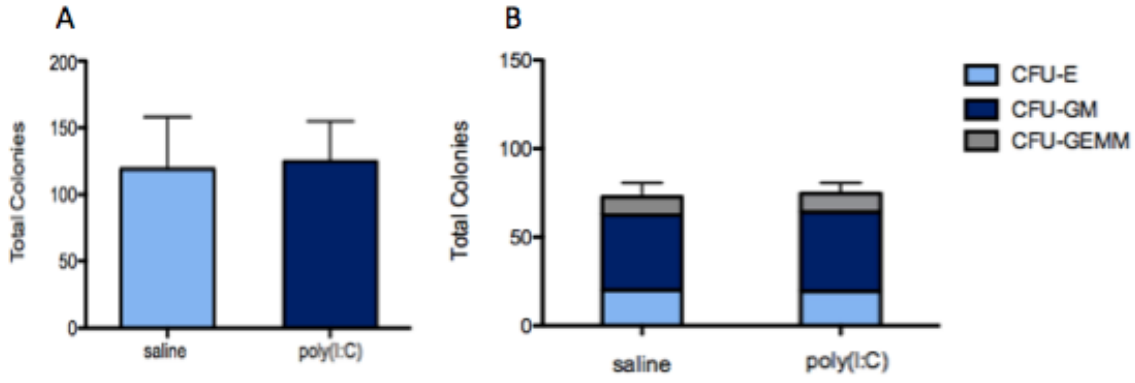

Figure 8. (A) Total Myeloid Colonies of Fetal Liver Primary Cells In Vivo Day 5. (B) Total Myeloid Colonies of Fetal Liver Primary Cells In Vivo Day 12

GATA Fetal Liver	**E13.5**	**E15.5**
HSC	same	Same
CD34	Same	Poly(I:C) up
c-Kit	Same	Same
CD34 high c-Kit high	Same	Same
GATA Placenta		
HSC	Poly(I:C) up	Same
CD34	Saline up	Saline up
c-Kit	Poly(I:C) up	Saline up
CD34 high c-Kit high	Saline up	Saline up

IL-7Rα Fetal Liver	**E13.5**	**E15.5**
HSC	same	same
CD34	Poly(I:C) up	Saline up
c-Kit	Poly(I:C) up	Saline up
CD34 high c-Kit high	same	same
IL-7Rα Placenta		
HSC	Saline up	Poly(I:C) up
CD34	N/A	N/A
c-Kit	N/A	Poly(I:C) up
CD34 high c-Kit high	N/A	Saline up

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Appendix B

Effects of maternal immune activation on early fetal brain cytokine responses

Elaine Y. Hsiao and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA 91125

Experiments were conducted by Elaine Y. Hsiao and will contribute to a future manuscript on the role of IL-6 in fetal brain development in MIA offspring

Introduction

In considering the mechanism underlying how maternal immune activation (MIA) leads to behavioral and neuropathological features of autism and schizophrenia in the adult offspring, the cytokine IL-6 is a key target. It was previously shown that IL-6 is necessary for mediating the effects of MIA; that is, injection of poly(I:C) into pregnant IL-6 KO mice leads to normal behavioral development in the offspring. Similarly, co-injection of poly(I:C) with a neutralizing antibody against IL-6 into wild-type pregnant mice also blocks the effects of poly(I:C) on fetal development. Furthermore, recombinant IL-6 injection into pregnant mice is sufficient to induce MIA-associated changes in offspring behavior. Thus, tracing IL-6 action shortly after maternal poly(I:C) injection offers the opportunity to elucidate the early molecular mechanisms that trigger downstream events leading to autism and schizophrenia-related endophenotypes in the offspring.

In chapter 6, we evaluate the role of IL-6 in the placenta shortly after MIA. We find that IL-6 is dramatically increased, at levels 17-fold greater than those seen in non-MIA controls. Furthermore, we demonstrate that IL-6 plays an important role in regulating endocrine function in the placenta in response to maternal poly(I:C) injection, altering levels of hormones and lactogens that play key roles in embryonic development.

Another potential site of IL-6 action, however, is in the fetal brain itself. Some reports point to the ability of maternal cytokines to cross the placenta and enter the fetus directly. Also, it is possible that the fetal brain itself responds to MIA by production of cytokines. Here we evaluate fetal brains from embryos of mothers injected with poly(I:C) versus controls to assess cytokine production shortly after MIA. Specifically, we utilize

qRT-PCR to detect IL-6 transcript in RNA derived from whole fetal brain lysate. We further utilize Luminex multiplex arrays to detect cytokine protein, including IL-6, from whole fetal brain lysate.

Results

IL-6 expression is induced in the fetal brain shortly after maternal poly(I:C) injection.

Fetal brains from MIA mothers experience a significant upregulation of IL-6 transcript by 3 hours post maternal poly(I:C) injection (Figure 1). This induction is transient, decreasing to levels that are not statistically different from those seen in saline controls by 6 hours post injection. Similarly, there is no significant difference between poly(I:C) and saline control embryos in the level of fetal brain IL-6 expression at 24 hours post maternal injection. Similar induction of IL-6 is observed at the protein level in poly(I:C) fetal brains compared to controls, as assessed by Luminex multiplex cytokine array (Figure 2). Overall, that IL-6 is induced in the fetal brain itself, albeit transiently, after maternal poly(I:C) injection indicates that IL-6 action directly in the fetal brain may manifest is detrimental downstream consequences on neurodevelopment.

IL-4 is dramatically induced in the fetal brain shortly after maternal poly(I:C) injection.

Interestingly, in addition to IL-6, the cytokine IL-4 is dramatically upregulated in the fetal brain at 3 hours post matmernal poly(I:C) injection as measured by Luminex array (Figure 2). In addition, this induction requires maternal IL-6 signaling as indicated by the lack of IL-4 induction in fetal brain when poly(I:C) is maternally injected into IL-6 knockout mice. Furthermore, basal levels of fetal brain cytokines are dependent on

maternal IL-6 signaling (Figure 2). There is a significant decrease in levels of all cytokines detected by Luminex array in fetal brains from poly(I:C) injected IL-6 KO mothers versus those detected in fetal brains from poly(I:C) injected WT mothers.

Discussion

We show here that shortly after maternal poly(I:C) injection, IL-6 is induced not only in the placenta, but in the fetal brain as well. The fetal brain induction of IL-6 (less than 2 fold that seen in controls) is considerably mild compared to that seen in poly(I:C) placentas (17-fold that seen in controls). In addition, the IL-6 induction in the fetal brain is more transient, lasting between 3 and 6 hours post maternal poly(I:C) injection, whereas the placental response to poly(I:C) leads to IL-6 induction that lasts over 24 hours post injection. It will be interesting to evaluate whether fetal brain IL-6 induction requires a placental IL-6 response.

We also demonstrate here that maternal IL-6 is critical in regulating fetal brain cytokine induction and as well as basal fetal brain cytokine levels. Thus, it is likely that maternal IL-6 reflects the pool of IL-6 that is particularly important in mediating MIA effects on offspring development. Further studies utilizing conditional IL-6R knockout mice will be necessary to confirm this notion.

Methods

Generation of animals.

Female C57BL/6N mice (Charles River; Wilmington, MA) were obtained from the Caltech breeding facility and housed under standard laboratory conditions. Mice were

mated overnight and the presence of a vaginal plug on the following morning was noted as day E0.5.

IL-6 KO mice.

IL-6 KO mice, strain B6.129S2-IL6tm1Kopf/J, were obtained from Jackson Laboratory (Bar Harbor, ME). IL-6 -/- females were mated with IL-6 +/+ males.

MIA.

Pregnant C57BL/6J or IL-6 KO mice were injected on E12.5 with saline, poly(I:C), or recombinant IL-6 (rIL-6). For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO) was freshly dissolved in saline and administered i.p. at 20 mg/kg based on the weight of the poly(I:C) itself, not including the total weight of the potassium salts. Control mice were injected with saline alone at 5 ml per gram body weight. *Measurement of fetal brain cytokines*. After injection on E12.5, wild type (WT) and IL-6 KO mice were sacrificed at 3 hours post injection by an overdose of sodium pentobarbital (Nembutal). Fetal brains were microdissected from the embryo and washed in PBS prior to snap-freezing in liquid nitrogen and storage at -80°C. To generate cell lysates, each fetal brain was placed in 1 ml of cell lysis buffer (50 mM Tris-HCl (pH 7.4) with 0.6 M NaCl, 0.2% Triton X-100, 1% BSA, and 1 EDTA-free protease inhibitor cocktail tablet/10 ml buffer) (Roche Applied Sciences; Indianapolis, IN). Each tissue was homogenized on ice using a syringe fitted with an 18G needle and then sonicated for 5 seconds at 10 mV. Total protein was measured by BCA assay (Thermo Scientific; Rockford, IL) according to the manufacturer's instructions. For cytokine profiling, mouse

20-plex cytokine arrays (Invitrogen) were run on the Luminex FLEXMAP 3D platform by the Clinical Immunobiology Correlative Studies Laboratory at the City of Hope (Duarte, CA).

Measurement of fetal brain gene expression by real-time PCR.

Fetal brains were quickly dissected from the embryo and washed in PBS prior to preservation in 1 ml TRIzol solution (Invitrogen; Carlsbad, CA). Tissues were passed through an 18G needle and sonicated for two rounds of 3 seconds at 10 mV separated by incubation on ice. Homogenates were processed by chloroform extraction and washing with 70% ethanol according to standard procedures. RNA was further purified by applying the cleared lysate to an RNeasy mini column (Qiagen; Valencia, CA), and an on-column DNA digestion was performed according to the manufacturer's protocols. Samples were assayed using the 2100 Bioanalyzer (Agilent Technologies; Santa Clara, CA) and confirmed to contain high integrity RNA (RIN > 8). 5 mg RNA per sample was reverse-transcribed using the iScript cDNA synthesis kit (Biorad; Hercules, CA). Resultant cDNA was purified using a PCR purification kit (Qiagen) and eluted in 50 ml PCR-grade water (Roche). Gene expression was measured using SYBR Green master mix with Rox passive reference dye (Roche) on the ABI 7300 Real Time PCR system. Target gene expression was normalized against beta-actin transcript, and data are expressed as ratios of gene expression of poly(I:C) to saline samples. The primers used were adapted from the Primerbank database (Spandidos et al., 2010).

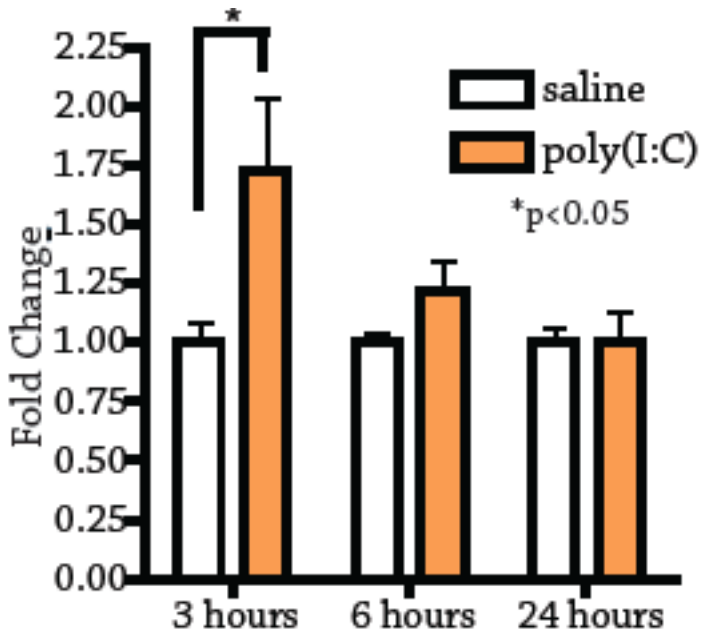

Figure 1. MIA elevates IL-6 expression in the fetal brain. At 3 hours post injection, fetal brains from mothers injected with poly(I:C) exhibit elevated levels of IL-6 mRNA compared to saline controls. This IL-6 induction is transient, as no significant differences are seen in fetal brain IL-6 levels at 6 hours and 24 hours post injection in E12.5 poly(I:C) versus saline offspring.

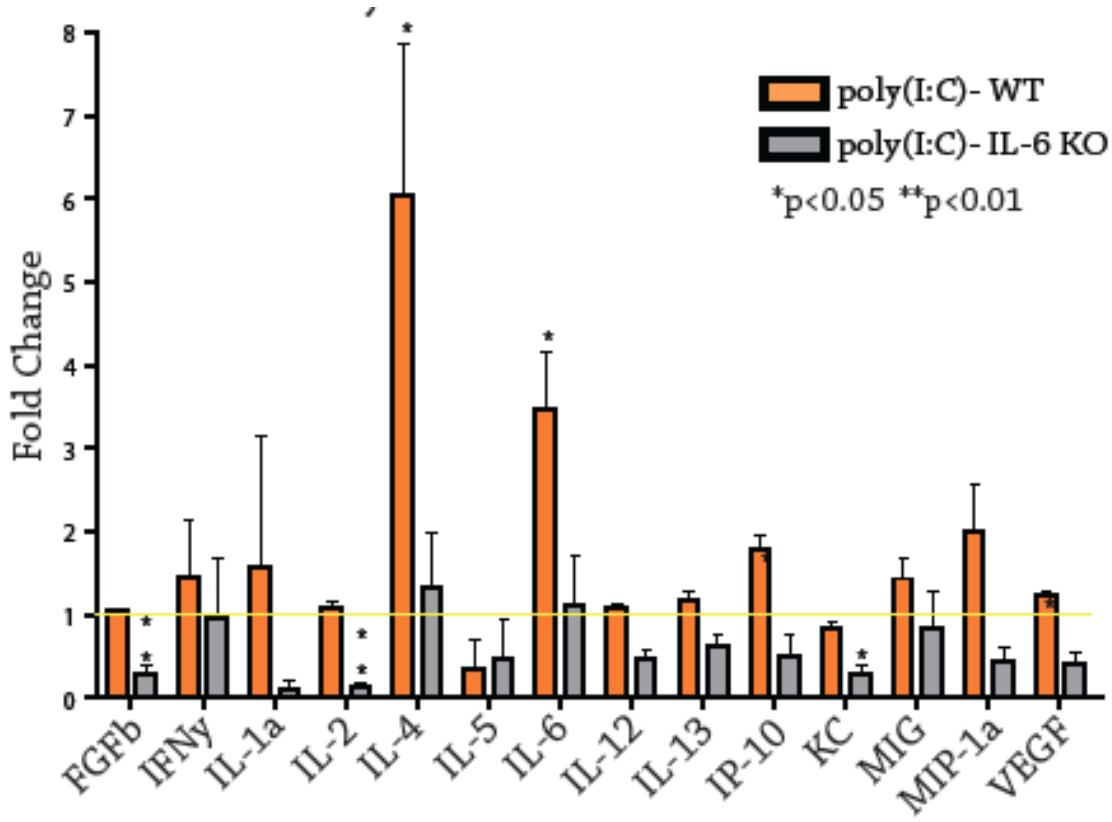

Figure 2. MIA elevates IL-4 and IL-6 protein in the fetal brain, which is dependent on maternal IL-6 signaling. At 3 hours post injection, fetal brains from offspring of poly(I:C)-injected mothers exhibit significantly elevated levels of IL-4 and IL-6 protein compared to controls. This induction was dependent on maternal IL-6 signaling, as cytokine levels are dramatically reduced in offspring of IL-6 KO mothers injected with poly(I:C).

Appendix C

Additional immune characterization of adult offspring of mothers exposed to gestational immune activation

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Experiments were conducted by Elaine Y. Hsiao as additional experiments relevant to the publication presented in Chapter 11

Introduction

Findings presented in chapter 11 demonstrate that maternal immune activation (MIA) induces long-term changes in offspring immunity. The persistent abnormalities are characterized by systemic deficits in T regulatory cells, hyper-active production of IL-6 and IL-17 by CD4+ T cells and increased levels of Gr-1+ monocytotic and neutrophilic cells. To evaluate whether MIA offspring exhibit additional changes in overall B cell and NK cell function, we measure levels of major immunoglobulin isotypes in sera of adult poly(I:C) versus control offspring and we evaluate cytotoxicity by NK cells isolated from spleens of poly(I:C) versus control offspring. In addition, we characterize levels of splenic memory CD4+ and CD8+ T cell subtypes. We further demonstrate that adult MIA offspring do not display primary changes in regional microglial abundance or activation. Here we explore whether fetal brains exhibit changes in microglial levels or status.

Results

There is no difference in levels of major immunoglobulin subtypes in sera from adult MIA offspring compared to controls.

Adult offspring of poly(I:C)-injected mothers display levels of serum IgM, IgG, IgA and IgE that are comparable to those observed in adult offspring of saline-injected mothers (Figure 1). This suggests no major change to B cell function.

There is no difference in cytotoxicity of NK cells isolated from spleens of adult poly(I:C) versus saline offspring.

NK cells derived from spleens of MIA offspring display similar levels of cytotoxicity against Yac-1 murine lymphoma cells in culture as compared to those derived from spleens of saline offspring (Figure 2). This suggests no primary abnormality in NK cell reactivity or function.

Adult MIA offspring exhibit decreased levels CD4+ effector memory cells and increased levels of CD4+ naïve T cells.

Interestingly, adult offspring of poly(I:C)-injected mothers display selective decreases in CD4+ CD44hi CD62Llo effecter memory cells and increased levels of CD4+ CD44lo CD62Lhi naïve T cells, but no difference in central memory cells (Figure 3). Altered levels of CD4+ and CD8+ memory subtypes are also observed in MLN cells from poly(I:C) versus saline offspring. In contrast, there is no significant difference in the level of CD4+ $CD44^{hi}$ $CD62L^{hi}$ central memory cells, or splenic CD8+ $CD44^{hi}$ $CD62L^{lo}$ effector memory cells, CD8+ $CD44^{hi}$ $CD62L^{hi}$ central memory cells, or CD8+ $CD44^{lo}$

CD62L^{hi} naïve T cells. Overall, MIA leads to abnormal distribution of CD4+ memory cells in adult offspring, consistent with reports of altered T cell memory subtypes observed in autistic individuals (Ferrante et al., 2003; Gupta et al., 1998). This indicates that primary alterations are observed in CD4+ T cells from MIA offspring, consistent with findings of decreased CD4+ T regulatory cells and hyper-responsiveness of CD4+ T cells to stimulation.

There is no significant difference in levels of fetal brain CD11b+ cells in response to poly(I:C).

To examine whether poly(I:C) offspring exhibit evidence of early microglial activation or proliferation compared to controls, we isolated whole fetal brain and generated single cell suspensions. We further applied flow cytometry using the macrophage/microglia marker CD11b and early activation marker CD69 to examine brain immune status. There is no difference in total levels of CD11b+ cells from fetal brains of poly(I:C) versus control offspring on E12.5. In addition, there is no significant difference in CD69+ activation in these CD11b+ cells (Figure 4). This suggests that maternal poly(I:C) injection does not induce global changes in microglial levels or activation.

Methods

Mice.

C57BL/6J mice (Charles River; Wilmington, MA) were housed under specific pathogen-free conditions in Caltech's Broad animal facility. Mice were mated overnight and the

presence of a vaginal plug on the following morning was noted as day E0.5. All experiments were performed under the approval of Institutional Animal Care and Use Committee.

MIA.

Pregnant C57BL/6J mice were injected on E12.5 with saline or poly(I:C). For poly(I:C) injections, poly(I:C) potassium salt (Sigma Aldrich; St. Louis, MO) was freshly dissolved in saline at 4 mg/ml and administered i.p. at 20 mg/kg based on the weight of the poly(I:C) itself, not including the total weight of the potassium salt. Control mice were injected with saline alone at 5 ml per g body weight.

Detection of Serum Immunoglobulins.

Sera were harvested from adult poly(I:C) and saline offspring by cardiac puncture, and allowed to clot for 30 minutes in Vacutainer SST tubes (BD Biosciences). Tubes were spun at 1500 xg for 10 minutes, and serum was collected above the separator gel, aliquoted, and stored at -80°C. Aliquots were used for endpoint ELISA assays for mouse IgM, IgG, IgA, and IgE (Biolegend) according to the manufacturer's protocols.

NK cell cytotoxicity assay.

Splenic single cell suspensions were isolated as described above. CD49+ microbeads (Miltenyi Biotec) were used for positive selection of NK cells. 10^5 effector NK cells were incubated, 5:1, with DiO-labeled Yac-1 murine lymphoma cells (ATCC; Manassas, VA) for 1 hour at 37°C, alongside effector-only and target-only controls. Cells were then

counterstained with propidium iodide (PI) and assayed by flow cytometry according protocols from the LIVE/DEAD cell-mediated cytotoxicity kit (Invitrogen; Carlsbad, CA). Data presented are [[(% effector-killed cells (DiO+ PI+) - % spontaneously dead cells (DiO+ PI+) from effector-only control)] / % total DiO+ target cells] x 100.

Flow cytometry.

Splenic and MLN single cell suspensions were generated as described above. For brain suspensions, adult mice were sacrificed by CO_2 gas exposure and frontal cerebral cortex, cerebellum, and hippocampus were carefully dissected in ice cold PBS. Single cell suspensions were generated using the Neural Dissociation kit (Miltenyi Biotec) according to the manufacturer's protocol. For extracellular staining, cells were treated with anti-mouse CD16/CD32 Fc block (eBioscience) for 10 minutes on ice prior to staining in CBSS buffer with subsets of these antibody conjugates: Ter119-PerCP-Cy5.5, CD4-FITC, CD8-FITC, CD11b-APC (eBioscience), CD69-PE, CD62L-APC, CD44-PE (Biolegend; San Diego, CA). Samples were processed using the FACSCalibur cytometer (BD Biosciences) alongside appropriate isotype, single-stain, and unstained controls. Data were analyzed using FlowJo software (TreeStar Inc.; Ashland, OR) and presented as percent frequency of the parent population (non-erythroid (Ter119-) cells).

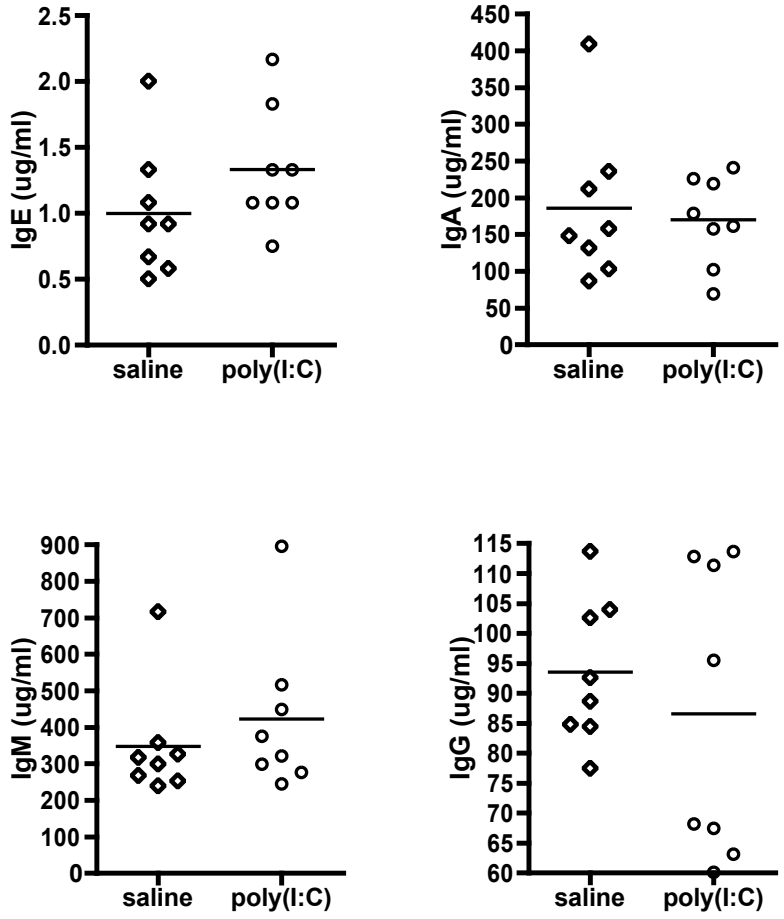

Figure 1. There is no significant difference in levels of total IgM, IgG, IgA and IgE in blood sera from adult poly(I:C) versus saline offspring [n=8 saline, 8 poly(I:C)].

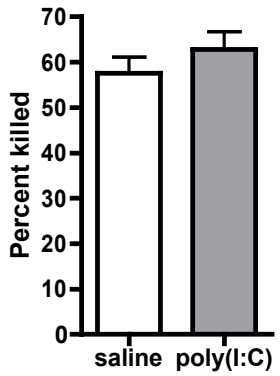

Figure 2. Splenic NK cells from adult poly(I:C) versus saline offspring exhibit similar levels of cytotoxicity against stimulating Yac-1 murine lymphoma target cells [n=4 saline, 4 poly(I:C)].

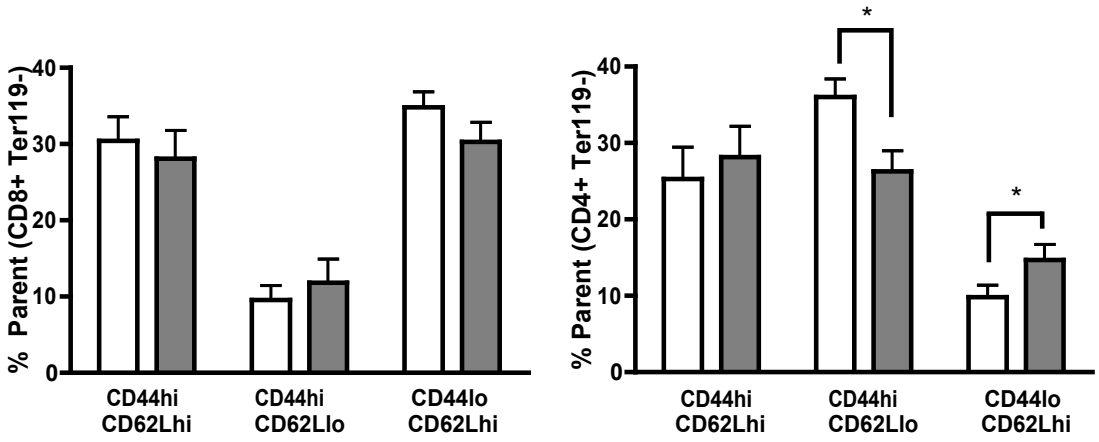

Figure 3. Compared to controls, spleens from poly(I:C) offspring exhibit decreased levels of CD4+ CD44hi CD62Llo effecter memory cells and increased levels of CD4+ CD44lo CD62Lhi naïve T cells (C, left). There is no difference in CD8+ memory cell subtypes from spleens of poly(I:C) versus saline offspring (C, right). [n=8 saline, 9 poly(I:C); *p<0.05]

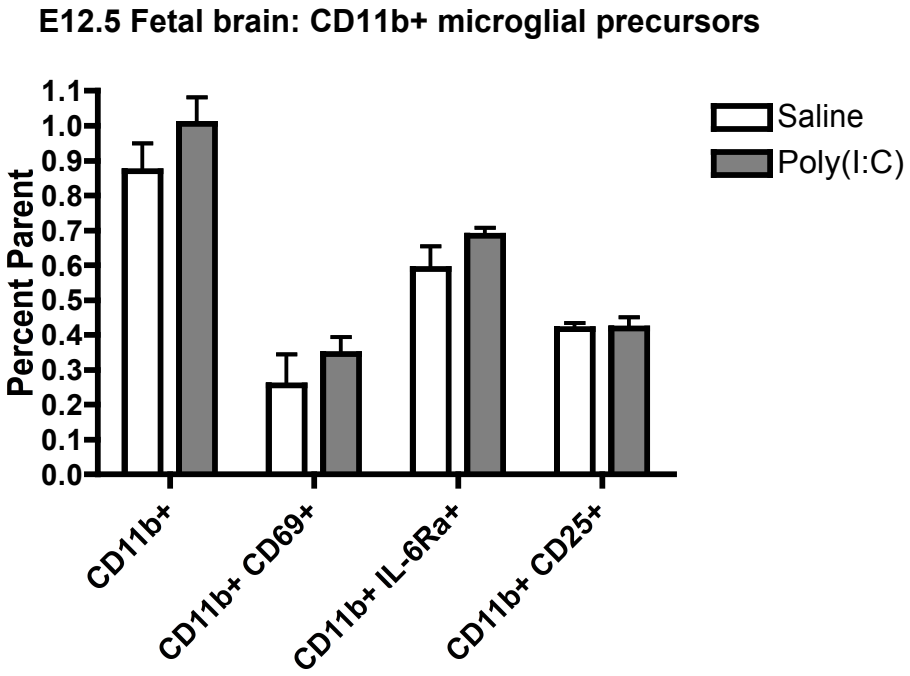

Figure 4. There is no difference in levels of CD11b+ cells in E12.5 fetal brains from poly(I:C) versus saline-injected mothers.

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Appendix D

Increased intestinal permeability is not sufficient to induce autism-related behaviors in mice

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Introduction

Autism is one of the five "Autism Spectrum Disorders (ASDs)" (the other four being Asperger's syndrome, Rett's Disorder, Childhood Disintegrative Disorder, and Pervasive Developmental Disorder-Not Otherwise Specified (PDD-NOS)). According to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, Text Revision (DSM-IV-TR) and International Classification of Diseases, Tenth Revision (ICD-10), the three key characteristics of autism are: impairments in language or communication, deficits in social interaction, and the increased tendency towards repetitive behaviors (White, 2003). CDC currently estimates that 1 out of 110 US children have an ASD, making this disorder more common than childhood cancer, juvenile diabetes, and pediatric AIDS combined.

The recent California Autism Twin Study conducted by Stanford University suggests that 58% of autism development risk can be attributed to non-genetic factors (Hallmayer et al., 2011). Within these non-genetic factors, the involvement of gastrointestinal symptoms is becoming more apparent. Historically, common gastrointestinal complaints reported in autism include chronic constipation and abdominal pain, and antibiotics have been seen to informally treat symptoms (Buie, 2010). Autistic children also frequently show food intolerance, which has led many parents to enlist a gluten-free or casein-free (casein is the major protein in cow's milk) diet (White, 2003). This "gfcf" diet commonly results in the amelioration of autistic behaviors: Lucarelli et al. (1995) showed marked behavioral symptom improvement in 36 autistic patients after 8 weeks on a milk-elimination diet and Knivsberg et al. (2002) showed normalizing of previously abnormal urinary peptide levels in autistic individuals

on the “gfcf” diet. Furthermore, autistic patients have displayed significantly eroded and more ulcerated intestinal epithelium compared to controls, in addition to increased intestinal permeability (Ibrahim, 2009). According to de Madistris et al. (2010), autistic patients on a reported “gfcf” diet showed reduced abnormal intestinal permeability compared to autistic individuals on an unrestricted diet.

Unfortunately, many of the current reports on the potential relationship between gastrointestinal symptoms and the onset of autism have not been able to be replicated or have had poor controls. The prevalence of recent interest in this research and identification of possible connections has led to the “leaky gut hypothesis”, which proposes the leakage of harmful toxins from the gut into the bloodstream, thus resulting in changes in the brain and behavior. More specifically, this hypothesis suggests that digestion products (such as those of wheat and milk) may be able to leak into the blood through leaky mucosal linings, initiating antigenic responses and possibly affecting the central nervous system, ultimately resulting in behavioral symptoms (White, 2003).

One of the most widely used mouse models in autism research today is the Maternal Immune Activation (MIA) model, in which an injection of synthetic double stranded RNA poly(I:C) in a pregnant mouse induces an immune response, and the resulting offspring display characteristics of autism as tested through behavioral assays (Patterson, 2011). It has been previously shown that poly(I:C) mouse exhibit leaky gut, providing further evidence for a relationship between gastrointestinal issues and the onset of autism.

Leaky gut is currently studied in mice through the use of dextran sulfate sodium (DSS), which when orally administered induces experimental colitis (inflammation of the large intestine) (Gaudio et al., 1999). Studies regarding DSS treatment have not

investigated any resulting behavioral abnormalities with respect to developmental disorders such as autism. Behavior studies that have been done include pain sensitivity assays which show that DSS-induced colitis correlates with mice exhibiting hyperalgesia in response to visceral capsaicin and restrain stress is related to the increased onset of DSS-induced colitis (Eijkelkamp et al., 2007, Milde and Murison, 2002).

In an effort to characterize the involvement of leaky gut in the onset of autism-like symptoms in a mouse model, this study seeks to examine the behavior of mice with DSS-induced colitis, or "leaky gut". By investigating behavior abnormalities using common autistic behavioral assays (pre-pulse inhibition, sociability, social interaction, open field test, marble burying assay) in non-poly(I:C) offspring with leaky gut, this research explores the sufficiency of leaky gut in development of the some of the core symptoms of autism (increased repetitive behavior and deficits in social interaction).

Results

Following DSS treatment, mice were monitored twice daily for weight gain (Figure 1). As expected, mice treated with 2% DSS showed diminished weight gain after approximately 1 week. 1%-DSS-treated mice did not show this diminished weight gain, and continued to gain weight normally, similar to the control mice. Mice were also scored for intestinal bleeding according to Table 1. Results show that 2%-DSS treated mice frequently displayed rectal bleeding, a hallmark of DSS-induced colitis (Figure 2).

To confirm existence of leaky gut in the DSS-treated mice, mice were gavaged with fluorescent compound FITC-dextran, and serum fluorescence was subsequently measured. Leaky gut assay results show that DSS-induced colitis yields a comparable

measure of serum fluorescence to that of poly(I:C) offspring (Figure 3). Furthermore, colon lengths of 2% DSS-treated mice were significantly shorter than control and 1% DSS-treated mice, which is a sign of successfully induced inflammation of the gut (Figure 4).

Real-time PCR was done on colon tissue to measure gene expression of various tight junction proteins. The proteins examined (clauidin 8, claudin 15, ZO-1, ZO-2, occludin) were chosen based upon previously seen differences in poly(I:C) gene expression. Colon tissue of 4-week-old mice showed no significant differences in tight junction protein transcript levels (Figure 5), despite existence of leaky gut. This finding suggests that leaky gut may be localized or that DSS-induced colitis results in the loss of proteins other than those examined in the current study.

Real-time PCR was again performed on adult mice colon tissue, and deficits were seen in both claudin 15 and occludin (Figure 6). These results parallel abnormalities seen in poly(I:C) transcript levels, and support the notion that leaky gut is localized to certain areas of the colon.

To visualize leaky gut in the DSS-treated mice, colon tissue was sectioned and stained with hematoxylin and eosin (H&E) markers (Figure 7). Control mice colon tissue show fully intact intestinal crypts. 1% DSS-treated mice display slightly disrupted crypts, whereas 2% DSS-treated mice clearly show atrophy. Images support molecular findings for the basis of leaky gut existence in 2% DSS-treated mice.

Behavioral assays were used to characterize the behavior of DSS-treated mice. Unlike poly(I:C) offspring behavior, the results of the DSS-treated mice assays showed no difference in PPI (Figure 8), social interaction (Figure 9), or marble-burying (Figure

10). The open field test showed no difference in the frequency of entering the center of the box, but a mild deficit in duration spent in center was seen (Figure 11). Upon closer examination, the deficit was seen to be significant in 2% males compared to control males, but not in females.

A leaky gut assay was performed on adult DSS-treated mice to confirm persistence of leaky gut into adulthood. Results showed that DSS-treated mice continued to exhibit decreased colon length and increased serum fluorescence (Figures 12, 13).

Discussion

DSS was first administered to PND 21 mice in 0%, 1% and 2% DSS by volume in drinking water to determine the optimal dosage. DSS is normally administered to adult mice, and monitored by observation of weight loss. Since autism is a developmental disorder (symptoms appear by the age of three in humans), and poly(I:C) mice were seen to exhibit leaky gut as early as 3 weeks, DSS was administered to 3 week old mice. Furthermore, since young mice are still growing, a diminished weight gain was noted for instead of weight loss. Weight monitoring, in addition to stool scoring, indicated successful induction of leaky gut in 2% DSS mice. Results were confirmed with the leaky gut assay: 2% DSS-treated mice showed a 1.6 fold increase in serum fluorescence compared to control mice; poly(I:C) offspring also show 1.6 fold increase in serum fluorescence compared to offspring of mothers injected with saline.

Real-time PCR was employed to examine loss of tight junction proteins in mice with leaky gut, using beta-actin as a “housekeeping gene”. The housekeeping gene is stably expressed, and varies only due to cell density differences, allowing for normalization

against potential transcript level differences due to tissue size, or other outlying factors. No significant differences were seen in tissue of the first leaky gut assay performed, suggesting the possibility that DSS-induced colitis involves the loss of tight junction proteins not examined in this study, or that the leaky gut is localized to certain areas of the colon that were not observed in the present samples. The second leaky gut assay done, on adult tissue, confirmed the persistence of leaky gut into adulthood and showed significant losses in claudin 8 and occludin proteins. This further suggests that leaky gut is localized and that the extensiveness of DSS-colitis-induced leaky gut and poly(I:C) leaky gut is similar, in that poly(I:C) offspring also show a loss in occludin and claudin 8 tight junction proteins.

The PPI assay, which measures the startle response of a mouse to a loud noise, showed no difference in the DSS-treated mice. Compared to controls, poly(I:C) mice display a deficit in PPI, but this was not seen in the DSS-treated mice, which suggests that leaky gut is not sufficient to produce this effect. Similar results were seen in the marble burying, sociability, and social preference assays, as differences seen in poly(I:C) mice were not replicated in DSS-treated mice. This further suggests that leaky gut is not sufficient for the onset of repetitive behavior and social interaction impairments. The open field center duration results displayed a mild deficit, and identified a sexual dimorphism, showing a potential correlation of DSS-treatment and development of anxiety in young mice. However, results obtained require substantiation through further research.

Overall, the findings of this study suggest that leaky gut is not sufficient to induce the core symptoms of autism in mice. Accordingly, continued research should be done in this

field to investigate if leaky gut is necessary for autism-like symptoms, which could be done by treating the leaky gut of poly(I:C) mice and observing if autistic behavior is ameliorated. It would also be interesting to research the molecular pathways behind why and how an injection of poly(I:C) in a pregnant mouse yields offspring with autistm-like symptoms. This research would shed light on the molecular basis for studies that have shown maternal immune system activation is correlated with the onset developmental disorders in the offspring.

Methods

Dextran Sulfate Sodium (DSS) Administration and Weight Monitoring

C57 mice from the Caltech breeding facility were housed under standard laboratory conditions. Mice from different litters were organized randomly into 0% DSS, 1% DSS, and 2% DSS treatment groups. Mice were orally administered DSS in 0%, 1%, and 2% concentrations by volume in drinking water and weight was monitored twice daily as a measure of ensuring mouse health. Stool samples were also scored according to Table 1 as measurement of leaky gut severity.

Leaky Gut Assay

Mice used for the leaky gut assay were separated from food and water for four hours before gavaging with FITC-dextran fluorescence at a concentration of 60ug FITC-dextran per 100g mouse (FITC-dextran stock solution was 100ug/ml). Four hours following gavaging, mice were sacrificed using CO2. Blood was collected using cardiac puncture and blood was allowed to clot in a vacutainer for 30 min before spinning down in a

centrifuge. Serum fluorescence was then measured against aliquots of a FITC-dextran standard solution using a Safire II spectrophotometer. Colon lengths were also measured following sacrifice and stored in paraformaldehyde for fixation before transferring to 30% sucrose overnight and flash-frozen in liquid nitrogen for storage at -80°C. Three 5mm sections of colon from each mouse were isolated for RNA extraction and placed in Trizol after washing in PBS and stored at -80°C.

Immunohistochemistry

Colons were dissected and fixed in 4% paraformaldehyde before storing overnight in 30% sucrose at 4°C. After equilibrating in the OCT (optimal cutting temperature) compound for 1 hour, colons were flash-frozen in liquid nitrogen and stored at -80°C. The tissue was sectioned into 12um slices using a microtome and then stained with hematoxylin and eosin to visualize gut morphology (hematoxylin stains for nuclei and eosin is a marker for cytoplasm). Stained slides were coverslipped using Vectamount aqueous solution and visualized under a microscope using bright light.

Measurement of Tight Junction Protein Levels through Real-Time PC

Colon tissue for RNA isolation was sonicated at 5-second intervals of 10mV until homogenized. By spraying the workspace and all involved materials with RNAse-Zap, RNases were eliminated, optimizing conditions for RNA extraction. The addition of chloroform to the homogenized samples in trizol and 4°C centrifugation following incubation and shaking yielded phase separation. Subsequent 70% ethanol (made with Diethylpyrocarbonate (DEPC) water) washes in RNeasy mini columns (Qiagen) allowed

for purification of RNA. Washes were performed according to standard manufacturer protocol and RNA was eluted using RNase-free water. Concentration of isolated RNA was then measured using a Nanodrop.

Using an iScript cDNA synthesis kit (composed of 5x iScript Reaction Mix, iScript Reverse Transcriptase, Nuclease-free water, and RNA template), 2ug of RNA per sample was reverse-transcribed into cDNA for real-time PCR (qPCR). Control samples with no reverse transcriptase, as well as with no RNA template were also created. Resulting cDNA levels were measured using a Nanodrop and cDNA was diluted in PCR water to a concentration of approximately 350ng/ul for qPCR. Gene expression was measured with a SYBR Green master mix (with Rox as a passive reference dye). Beta-actin was used as a housekeeping gene and transcript levels were measured for tight junction proteins claudin 8, claudin 15, ZO-1, ZO-2, and occludin.

Behavioral Assays

Five commonly used behavioral assays in autism-like symptoms diagnosis were employed to characterize the behavior of DSS-treated mice: pre-pulse inhibition (PPI), sociability, social interaction, open field test, and marble burying. Mice were tested for PPI at 6 weeks, sociability and social interaction at 7 weeks, marble burying and open field test at 8 weeks of age.

The PPI assay tests the startle response of a mouse to a loud noise. After 5 minutes of habituation to the testing chambers, mice are subjected to randomized blocks of no startle, startle (120db pulse), startle with a 5db prepulse, or startle with a 15db

prepulse. PPI is defined to be (startle stimulus only – (5 or 15 db prepulse + startle))/startle stimulus only.

The sociability and social preference assays measure for deficits in social interaction. In the sociability assay, the mouse is habituated in a three-chamber construct for 10 minutes. The mouse is then placed in the center chamber and given the choice to interact with a novel mouse or a novel toy. Its movement is tracked for 10 minutes; chamber frequency data describes the number of times the mouse enters the respective chamber, and contact frequency describes the number of times the mouse has contact with the toy or novel mouse. Chamber duration and contact duration delineate time spent in the respective chamber or interacting with the contact. The social preference assay tracks the mouse for 10 minutes, with a choice given between interacting with a familiar or unfamiliar mouse (of the same gender).

The open field test assay measures a mouse's anxiety and willingness to enter or spend time in the center of a box. Open field testing consists of a 10 minute habituation period of mice singularly placed in an empty rectangular box. Mouse movement is then tracked for 10 minutes, specifically noting for number of times the mouse enters the center of the box (frequency) and time spent in center of the box (duration).

The marble burying assay identifies repetitive behavior in mice. After 10 minutes of habituation in the testing cages, mice are given 18 marbles (arranged equidistantly in 3 columns by 6 rows) for 10 minutes. The number of marbles buried was noted such that a marble covered in >50% bedding was considered buried.

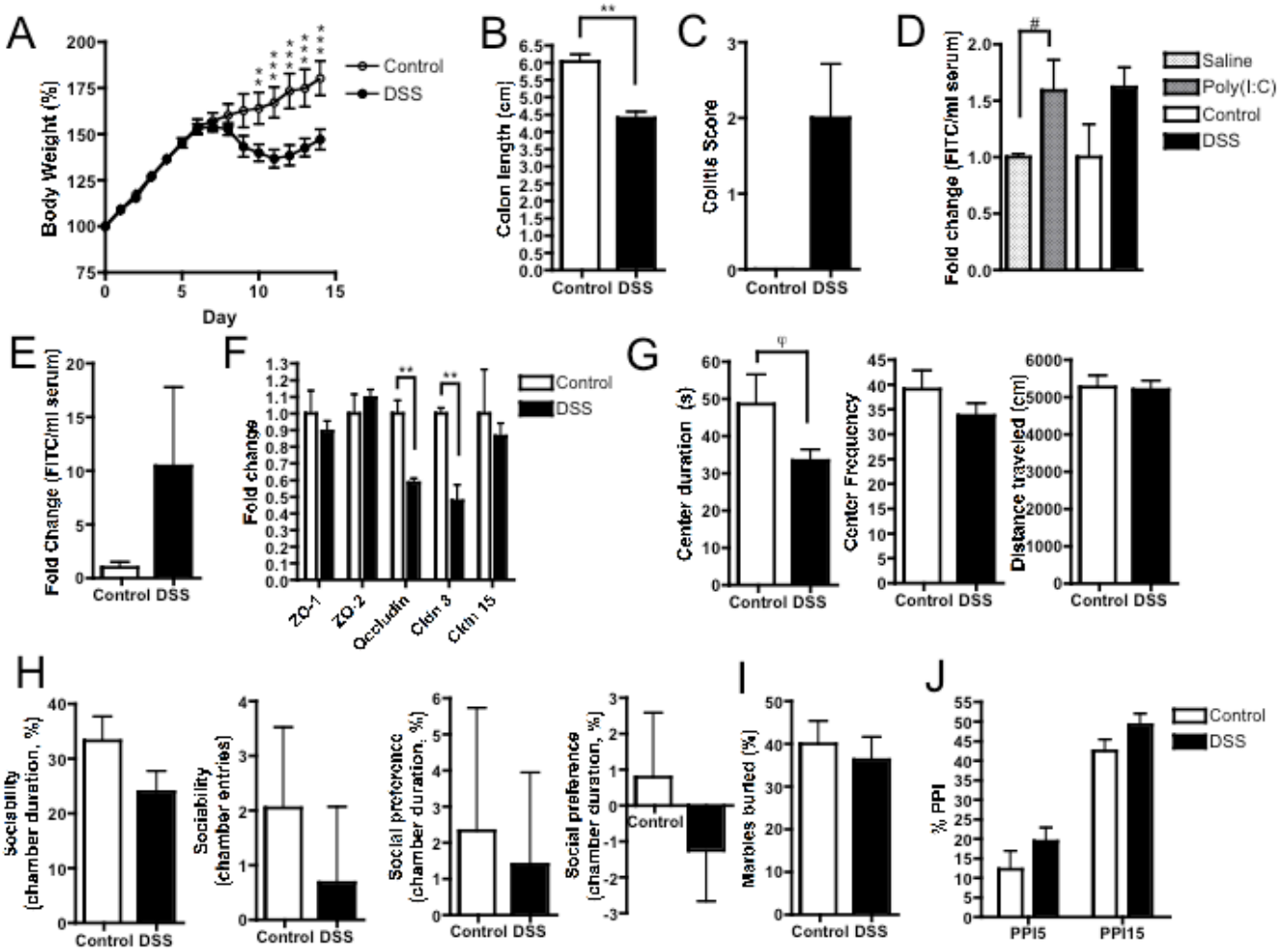

Figure 1. Early life treatment with 2% DSS induces mild colitis symptoms and leaky gut but no differences in autism-related behaviors. (A). Treatment of 3 week old mice with 2% DSS in water for 5 days leads to decreased weight gain versus wildtype controls. (B) DSS-treated mice exhibit decreased colon length and colitis-related symptoms (C). (D) 5 day treatment of 3 week old mice with 2% DSS leads to mild leaky gut symptoms similar to those seen in 3 week old poly(I:C) offspring, as measured by FITC-dextran translocation into the bloodstream after oral gavage. (E) 10 week old adult DSS-treated mice retain the increased intestinal permeability observed at 4 weeks of age. (F) Adult

DSS-treated mice exhibit decreased colon occludins and claudin 8 levels versus controls, similar to the phenotype observed in adult MIA offspring, as described in Chapter 12. (G) Adult DSS-treated mice display trending decreases in open field center entries, suggestive of increased anxiety-like behavior, but no differences in center duration of total distance traveled in the open arena. (H). DSS-treated mice do not show differences in social interaction behavior, stereotyped marble burying (I), or prepulse inhibition (J).

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Appendix E

The role of the commensal microbiota in the development of autism-related behaviors in mice

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Experiments were conducted by Elaine Y. Hsiao and Sophia Hsien, and will contribute to a future manuscript on the role of commensal microbes on behavioral development

Introduction

While commensal microbes are known to regulate a vast array of biological processes, including immune development, energy utilization and metabolism, they are only recently being appreciated for their important roles on the central nervous system. Commensal microbes play important roles in the production of neuroactive molecules, including neurotransmitters and neuropeptides, and in the development of complex behaviors, such as anxiety-like, emotional and nociceptive behavior. Modifying the composition of the microbiota in animal models affects circulating and brain levels of neurotransmitters such as serotonin and GABA, and germ-free mice which lack microbiota are known to exhibit several abnormal behaviors, including increased locomotion and decreased anxiety. Alterations in the composition of the microbial communities that inhabit us are further implicated in a variety of neurological and neurodevelopmental diseases, including multiple sclerosis, depression and autism.

In Chapter 12, we show that modulating the composition of the intestinal microbiota ameliorates autism-related behaviors in a mouse model of autism. We provide evidence that commensal bacteria of the microbiota can influence the gut-brain connection by modulating metabolites that alter behavior, and that in a mouse model of autism, a microbe-based therapy improves GI pathologies in conjunction with behavior. To further evaluate whether commensal microbes normally contribute to autism-related behaviors in wildtype mice, we assessed behavioral performance in germ-free versus conventionally colonized (specific pathogen free, SPF) mice in tasks measuring communication, stereotyped/repetitive behavior, and social interaction, among others.

Results

The commensal microbiota modulates the development of behaviors relevant to ASD

The microbiota is known to modulate a variety of behaviors, including motor, sexual, nociceptive and anxiety-like behaviors, but whether it also affects the development of behaviors relevant to ASD is unknown. We therefore tested GF and SPF mice for social interaction, stereotyped/repetitive behaviors, communication, pre-pulse inhibition and open field exploration to assess the core behavioral symptoms of autism along with additional ASD-associated behaviors. Based on findings that same-strain mice from different commercial vendors harbor distinct microbial populations, we utilized SPF C57Bl/6 mice from Taconic Farms (SPF-T) and from Charles River Laboratories (SPF-CR) for testing.

Consistent with previous reports, GF mice exhibit hyperactivity in the open field, as measured by increased total distance traveled and center entries, when compared to both SPF-T and SPF-CR mice (Fig 1A). This hyperactivity is similarly observed across all three trials (habituation, sociability and social preference) of social interaction testing. Interestingly, GF mice also display altered performance in a number of autism-related behavioral tasks. Adult male GF mice exhibit significantly fewer ultrasonic vocalizations in the presence of a novel female mouse (Fig 1B) than do SPF controls. Moreover, the individual calls emitted by GF mice are of significantly shorter duration compared to those from both SPF-T and SPF-CR mice suggesting a deficit in not only the quantity but also the quality of calls produced. This decrease in communication is not due to a lack of exploratory interest, since there is no significant difference between GF and SPF-CR mice in the time spent interacting with the novel mouse during the ultrasonic vocalization task (Fig 1C). Moreover, the decrease in communication observed in GF mice is not due

to confounding abnormalities in olfaction, as GF mice exhibit normal olfactory discrimination compared to SPF-CR controls (Fig 1D). In addition to altered vocalizations, GF mice also display disrupted sensorimotor gating as measured by decreased inhibition of an acoustic pre-pulse at 5 or 15 db above background noise (Fig 1E), with no difference in startle response intensity. This phenotype is consistent with symptoms observed in human autism, as well as other neurodevelopmental disorders such as schizophrenia.

To assess additional core symptoms of autism, we tested GF versus SPF-CR and SPF-T mice in repetitive/stereotyped behavior and social interaction. There is no difference between GF versus SPF mice in stereotyped marble-burying behavior (Fig 2A). However, GF mice engage in significantly elevated levels of self-grooming compared to SPF-CR, but not SPF-T mice (Fig 2C), highlighting an underlying difference in a repetitive/stereotyped behavior between SPF mice derived from Taconic Farms versus Charles River Laboratories that is statistically significant by Student's t-test (p=0.03) but not by one-way ANOVA with Bonferroni post-hoc test. An additional vendor-based difference is observed in social interaction testing: while mice typically display a preference for social novelty, as reflected by increased interest in an unfamiliar mouse versus a familiar mouse, SPF-T lack this social preference, exhibiting a notable preference for the familiar mouse rather than unfamiliar mouse (Fig 2D). There is no difference, however, between performance of GF, SPF-T or SPF-CR mice in the sociability task, which measures a mouse's preference to explore a social object (novel mouse) versus a non-social object (novel toy) (Fig 2B). Together these findings demonstrate that differences in microbial composition can lead to altered social,

stereotyped, communicative and sensorimotor behavior. These findings further suggest that disruptions to the normal microflora may initiate changes that manifest in alterations in behavioral development.

The commensal microbiota modulates the development of communicative behavior in diverse paradigms

We show in figure 1 that germ free mice emit dramatically fewer adult ultrasonic vocalizations of significantly shorter duration compared to both Taconic and Charles River SPF controls. To further evaluate communication-related behavior in germ-free mice we tested adult mice in the scent-marking paradigm. These tests measure the propensity for mice to engage in the placement of chemical signals used as a means to communicate with other mice. There was no difference in the level of scent marks produced by male GF versus SPF mice after one hour of habituation to a novel cage (Fig 3). This indicates no difference in scent marking response to novelty in the absence of stimulation. Interestingly however, GF males produce significantly fewer scent marks in response to both female urine and male urine (Fig 4), indicating a primary deficit in another mode of adult communicative behavior.

To gain insight into whether the communication abnormalities in GF mice compared to SPF controls are established early on during development, we assessed the level of USV production in pups from postnatal day 6 through 14 in response to brief maternal isolation. Remarkably, GF pups emit significantly more USVs after isolation from the mother compared to SPF controls (Fig 5). This increase in number of USVs produced corresponds to a great total duration of calls, but there is no difference in

average duration per single call, suggesting no overt differences in quality of USVs produced between GF and SPF mice. Overall, we find that GF mice exhibit a core abnormality in communicative behavior across several experimental paradigms, and that these impairments may be established by early postnatal development.

Effects of conventionalization on behavior of GF mice

To determine whether these behavioral abnormalities are reversible during adulthood, we conventionalized GF mice with either Taconic or Charles River microbiota and assessed behavioral performance. Mice were confirmed to be conventionalized by plating and qPCR (data not shown). However, there was no restoration in PPI or open field behavior (Fig 6). Interestingly, there was trending amelioration of adult USV behavior in response to adult conventionalization, but more studies are needed to confirm this phenotype. The inability to restore behavior is similarly seen with adult conventionalization with *B. fragilis*, indicating that effects *B. fragilis* alone on immunity or metabolism are not sufficient to alter behavior (Fig 7). Futhermore, *B. fragilis* monocolonized mice, born from *B. fragilis* monocolonized dams, also retain deficits in PPI and open field behavior (Fig 8). Interestingly, there is again a trending improvement in adult communicative behavior as measured by abundance and duration of vocalization. This phenotype, like that seen in adult conventionalized mice, requires further investigation.

To determine whether there is a developmental component to microbiota-mediated restoration of behavioral abnormalities in GF mice, we mated adult conventionalized males and females, to evaluate behavioral performance of ex-GF

offspring, which are born conventionalized from parent males and females that were conventionalized during adulthood. Ex-GF mice exhibit a statistically significant improvement in pup USV, but the effect is mild in that levels of pup USV in ex-GF still significantly differ form those observed in conventional SPF mice (Fig 9). As adults, ex-GF mice again retain deficits in PPI and open field behavior compared to SPF controls. These abnormalities are equivalent to those observed in adult conventionalized and GF mice (Fig 10). The adult ex-GF mice further retain deficits in scent marking behavior, at levels similar to those seen in GF mice (Fig 11). It will be interesting to assess whether adult USVs are impaired in these ex-GF mice. These measurements are in progress. Overall, these experiments suggest that adult USVs may be ameliorated by conventionalization and potentially reversible by microbiota manipulation during adulthood. However, that PPI and open field behaviors are not restored by conventionalization suggests that there may be underlying genetic alterations between GF and SPF mice of the same background strain but from different vendors. Both of these notions will be explored further in future studies.

Methods

Generation of animals.

Specific pathogen-free (SPF) C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, MA) or Taconic Farms and housed under standard laboratory conditions in Caltech's animal facility. Germ-free (GF) mice were obtained originally from University of North Carolina and housed in sterile isolators in Caltech's animal facility.

Behavioral testing.

At 6 weeks of age, offspring were behaviorally tested for PPI; at 7 weeks, for open field exploration, at 8 weeks, for self-grooming and marble burying, at 9 weeks for social interaction and at 10 weeks for scent marking and/or adult ultrasonic vocalizations, according to methods described by Hsiao et al., 2011 and Malkova et al., 2012. Additional details are provided in the supplementary methods. All equipment was sterilized with Process NPD and 70% ethanol before and after testing. GF mice were removed from the isolators at least two days prior to PPI testing and allowed to habituate to standard housing rooms. Immediately after PPI testing, GF mice were kept on water containing erythromycin and gentamycin to maintain GF status.

Conventionalization.

Fecal pellets were collected from SPF-Taconic or Charles River animals and homogenized in sodium bicarbonate. 100ul of the slurry was used for oral gavage into GF animals. On the same day, GF mice were transferred into a novel housing cage containing dirty bedding from respective SPF Taconic or Charles River mice.

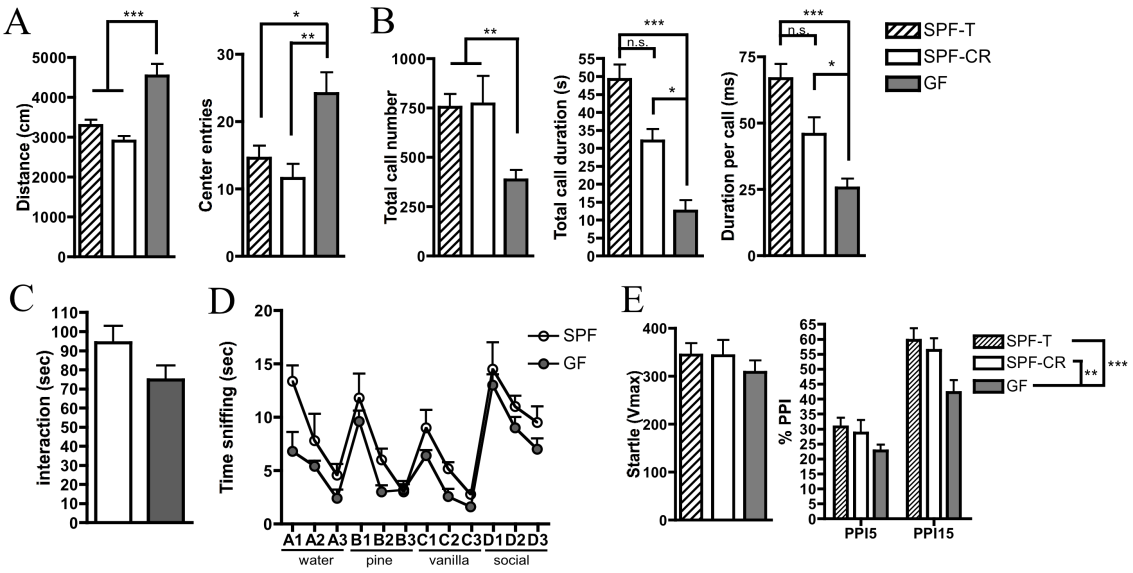

Figure 1. Germ-free mice display abnormal anxiety-like, sensorimotor and communicative behavior.

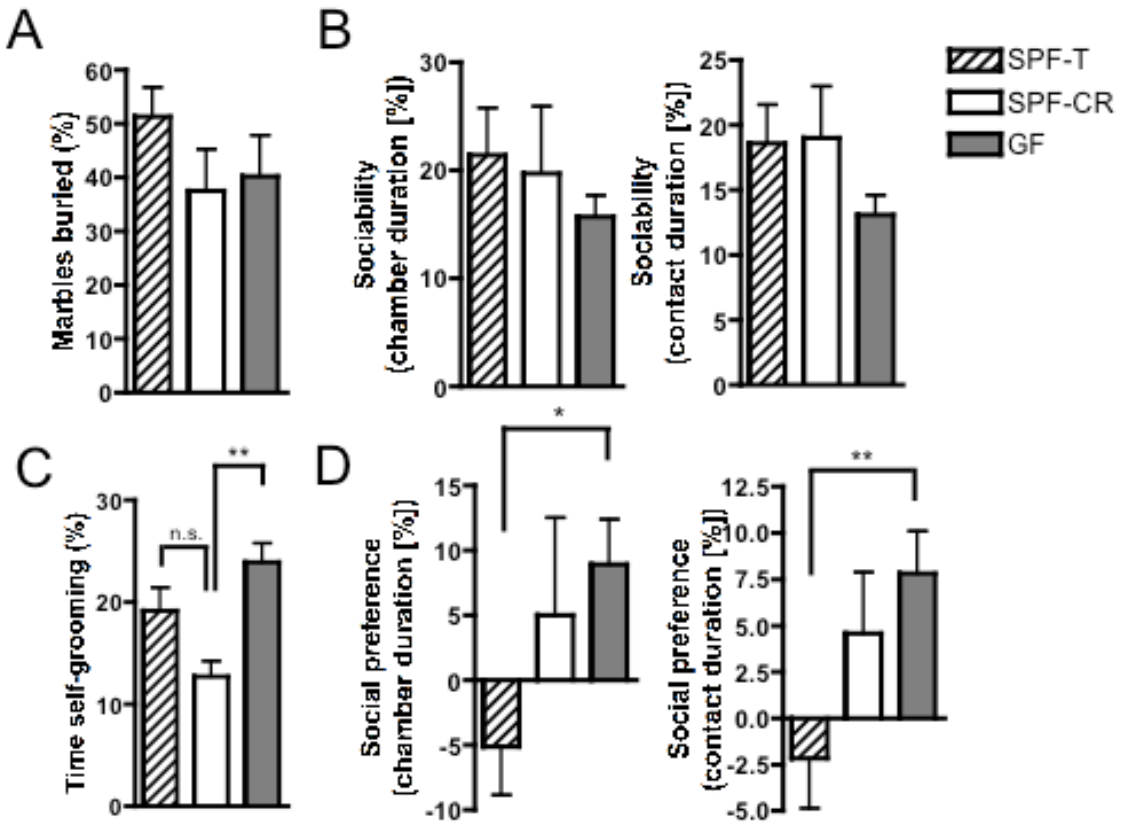

Figure 2. GF mice do not consistently exhibit altered stereotyped/repetitive or social interaction behavior.

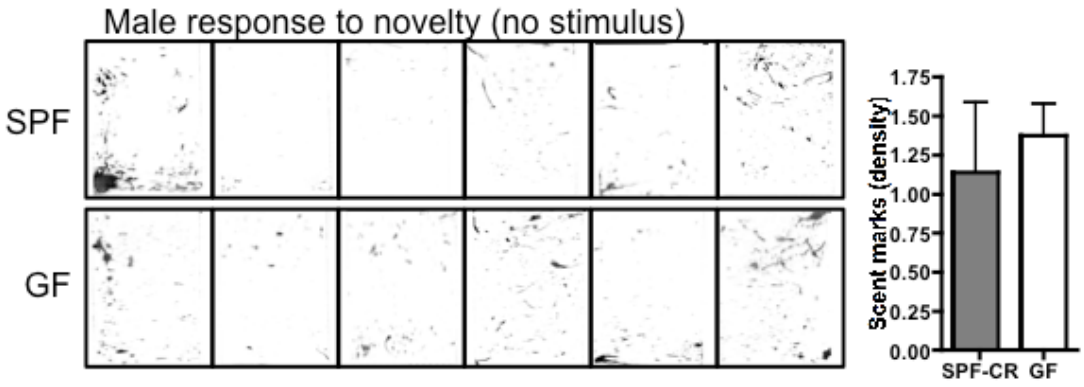

Figure 3. There is no difference in scent marking levels in response to spatial novelty in GF versus SPF mice.

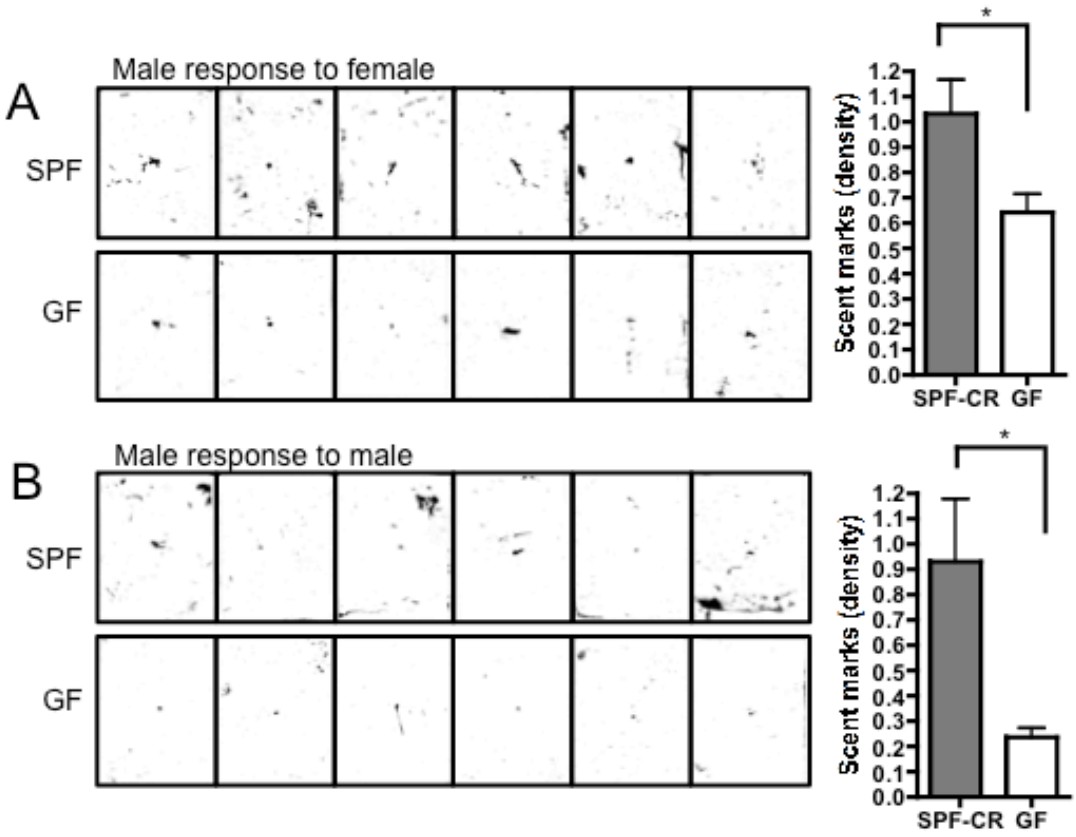

Figure 4. GF mice exhibit significant deficits in scent marking in response to female or male urine.

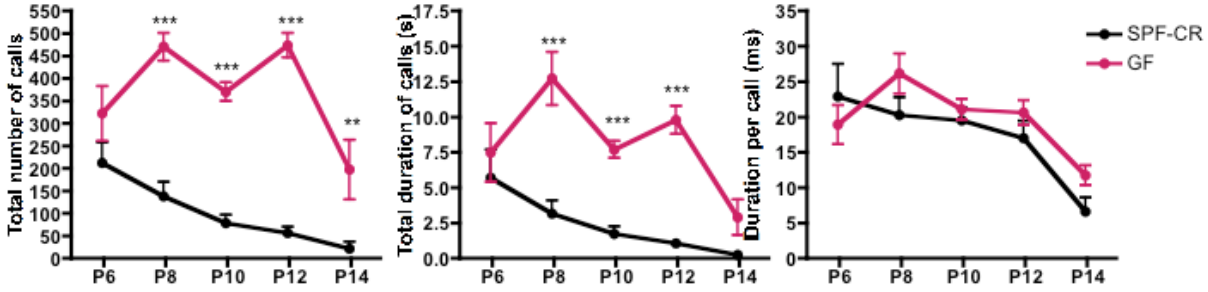

Figure 5. GF pups exhibit significantly elevated levels of ultrasonic vocalizations in response to brief maternal isolation.

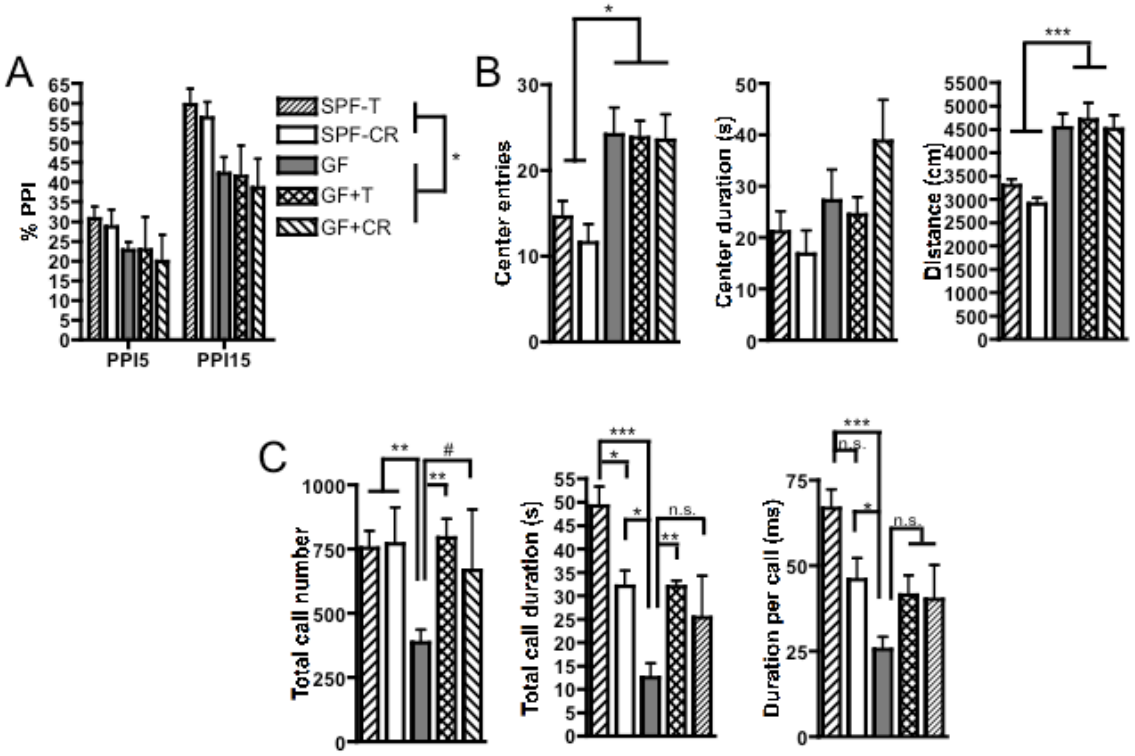

Figure 6. Adult conventionalization has no significant effect on prepulse inhibition or open field behavior, but elicits trending improvement of adult USV behavior.

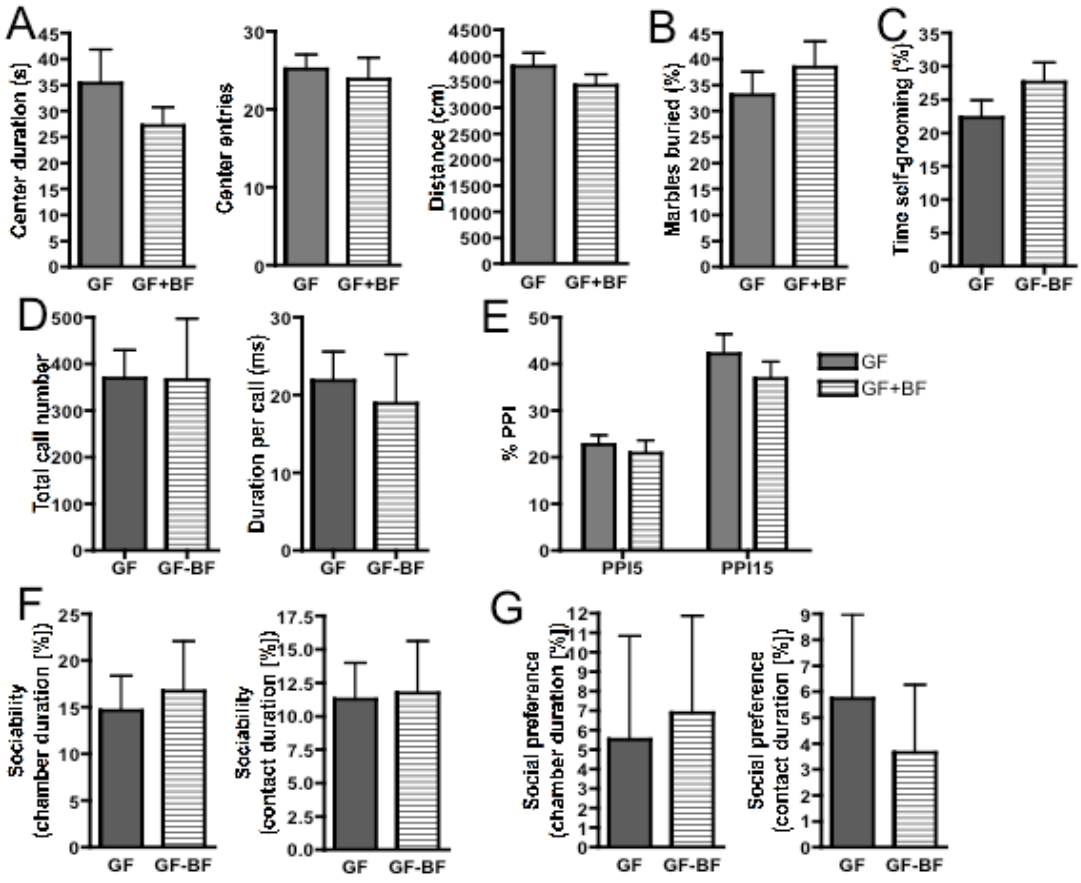

Figure 7. Adult monocolonization with *B. fragilis* has no significant effect on the behavior of GF mice.

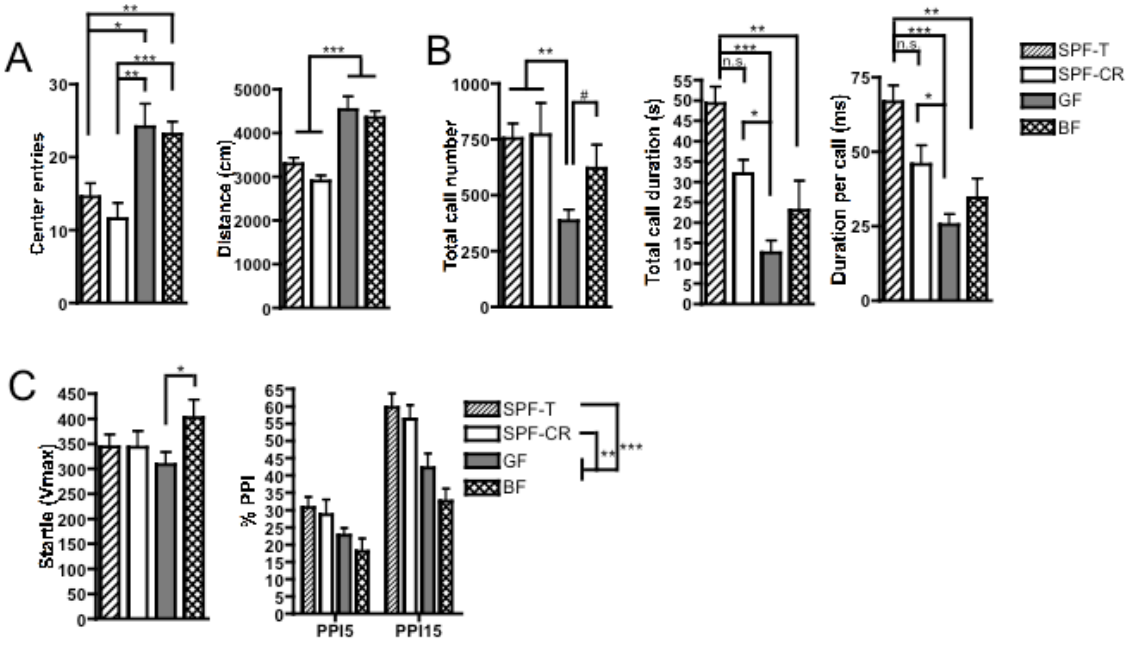

Figure 8. *B. fragilis* monocolonized mice exhibit behavioral abnormalities similar to those seen in GF mice, but trending improvement in adult USV.

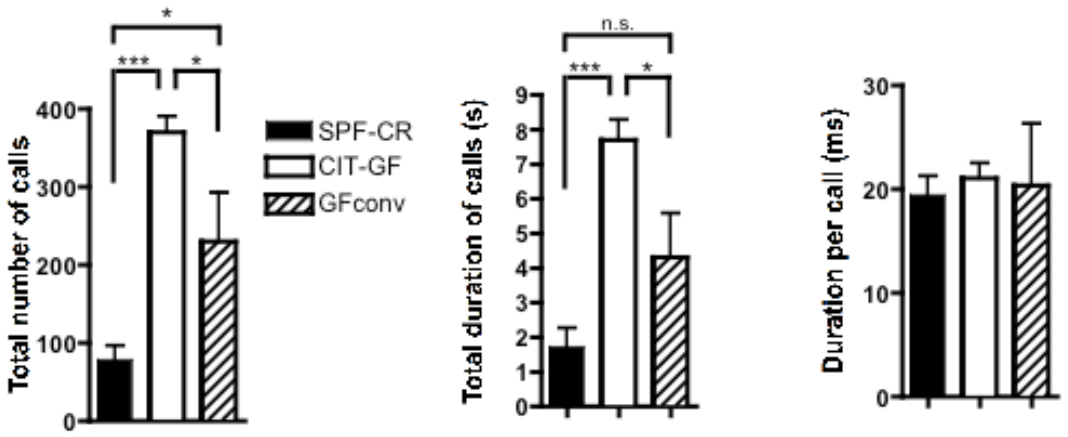

Figure 9. Conventional offspring of adult conventionalized mice exhibit mild improvements in pup USV in response to brief maternal isolation.

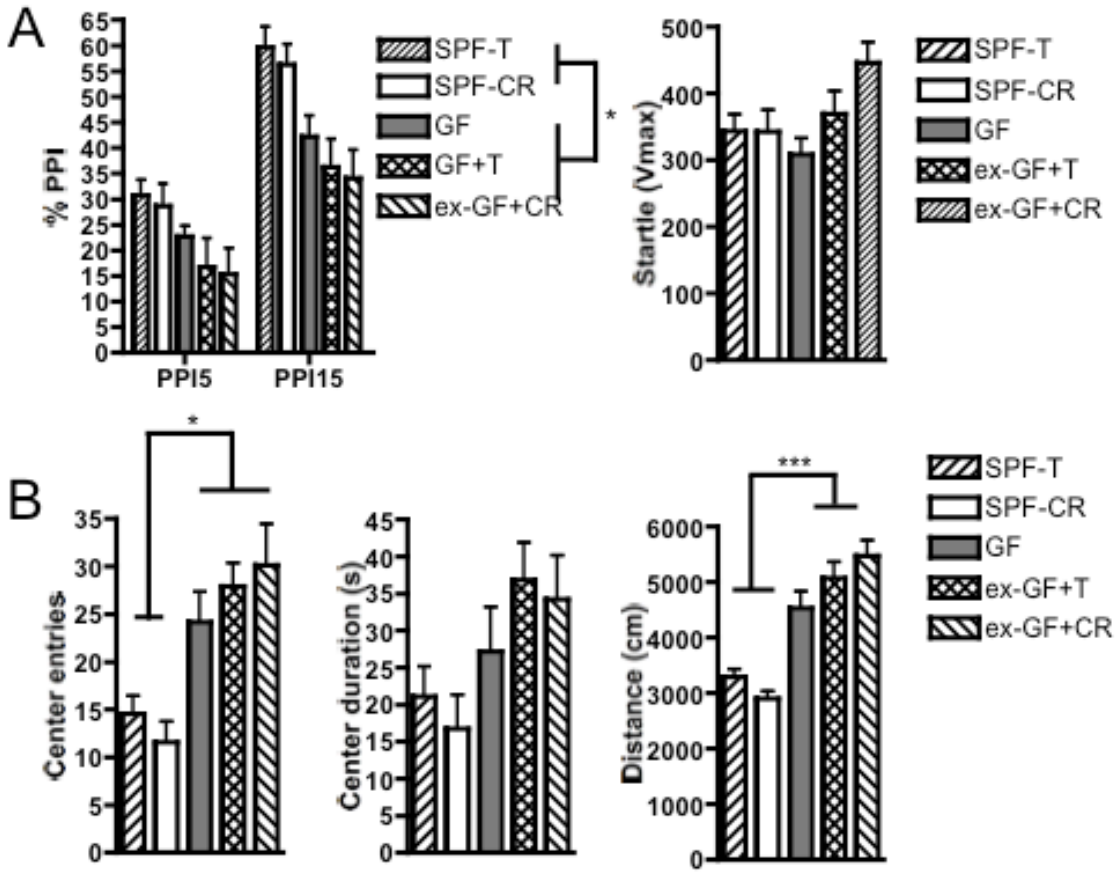

Figure 10. Conventional offspring (ex-GF) of adult conventionalized mice do not exhibit restored prepulse inhibition or open field behavior.

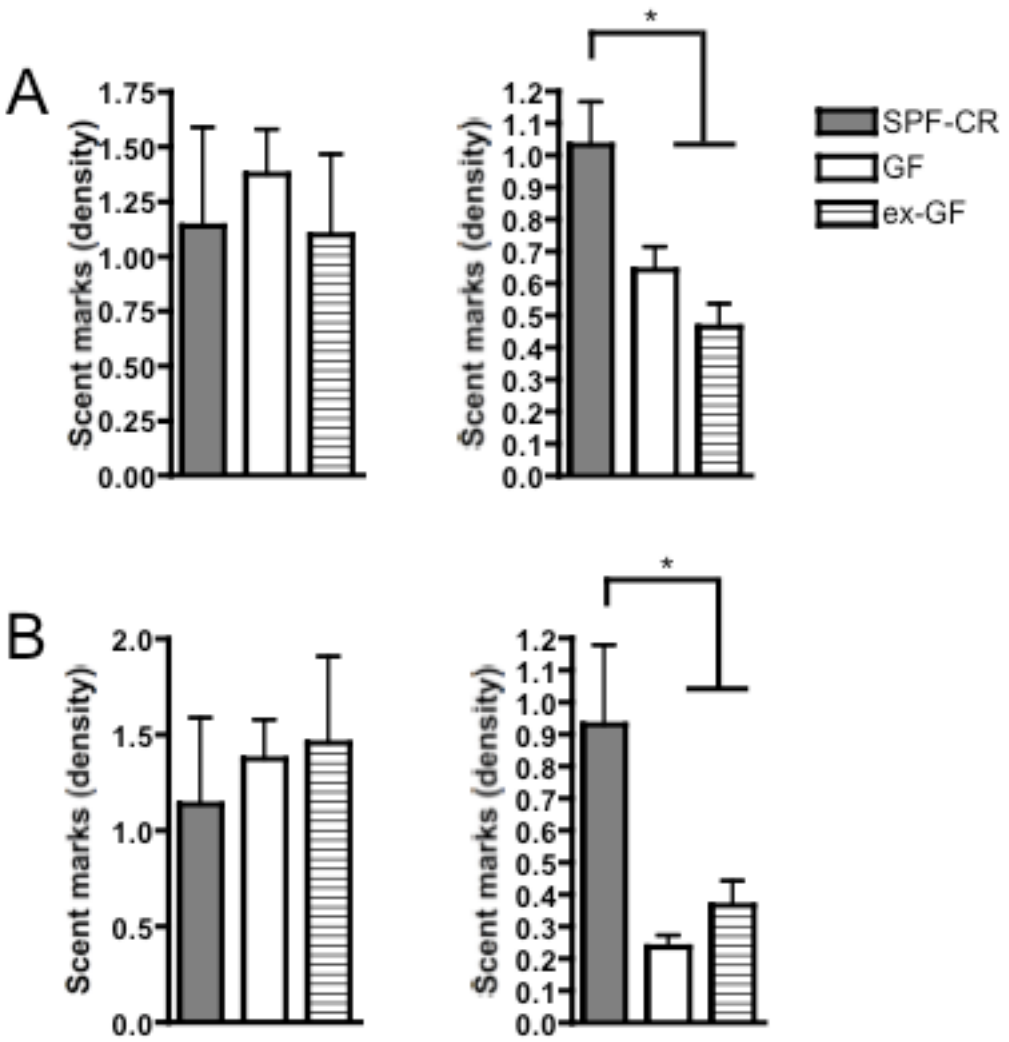

Figure 11. Conventional offspring (ex-GF) of adult conventionalized mice do not exhibit restored scent marking behavior.

Appendix F

Assessing a potential gene-environment interaction between the beta-2-microglobulin and maternal immune activation

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Experiments were conducted by Elaine Y. Hsiao and will contribute to a future manuscript on the role of IL-6 in fetal brain development in MIA offspring.

Introduction

Autism and schizophrenia are neurodevelopmental disorders for which the precise causes are unknown. Both exhibit a high degree of genetic heritability; however, environmental factors have been identified that contribute to the development of each disorder. Of these, maternal immune activation (MIA) is of particular interest given the several large epidemiological studies indicating that maternal bacterial or viral infection during the first or second trimesters of pregnancy increase the risk for autism and schizophrenia in the offspring. As described in Chapters 2-4, several mouse models of MIA have been developed that recapitulate core behavioral and neuropathological features of both disorders.

Interestingly, several immune-related susceptibility genes have been identified by genetic association and linkage studies of autistic and schizophrenic individuals. In particular, human HLA variants and short nucleotide polymorphisms, leading to impaired function of specific isotypes of human major histocompatibility complex (MHC), have been replicated as susceptibility genes. MHCI is canonically expressed on cells of the innate immune system, used to present unique antigenic components to T cells of the adaptive immune system. Recent studies reveal that MHCI also plays a key role in the brain, being expressed by neurons and glia alike. Neurons lacking MHCI display critical deficits in synaptic transmission, leading to impaired long term potentiation.

To explore a potential gene and environmental interaction between MHC and MIA, we ask whether MHC-deficient mice exhibit potentiated responses to the environmental factor MIA. In particular, we utilize beta-2-microglobulin (b2m) knockout

mice which lack a key component of mouse MHCI, in combination with maternal poly(I:C) injection mimicking viral infection.

Results

Heterozygous b2m KO mice exhibit expected responses to maternal poly(I:C) injection

In order to preserve as much as possible the maternal immune response to poly(I:C), we utilized pregnant b2m +/- mice mated to b2m +/- males. In wildtype mice, we typically observe that mothers injected with poly(I:C) on E12.5 display significant weight loss at 24 hours post injection, which restores to normal levels thereafter. We see a similar effect in pregnant b2m +/- mice, where maternal poly(I:C) injection leads to decreased maternal weight at 24 hours post injection (Figure 1). This indicates that b2m +/- mice experience a comparable immune response to poly(I:C), with associated sickness behavior and weight loss.

There is no effect of b2m genotype on MIA-induced deficits in pre-pulse inhibition. Adult MIA offspring of +/+, +/- and -/- b2m genotype were behaviorally tested starting at 6 weeks of age. In prepulse inhibition, there is no effect of b2m genotype on behavioral performance. However, the expected treatment effect of MIA on inducing deficits in PPI are observed regardless of genotypes (Figure 2).

No MIA treatment or b2m genotype effects are observed in open field anxiety or stereotyped marble burying behavior. That b2m offspring of any genotype exhibit expected defects in PPI behavior in response to MIA suggest that b2m +/- mothers

experienced expected responses to poly(I:C) induction. This is consistent with the expected maternal weight loss observed in b2m +/- mice after poly(I:C) injection. Interestingly, however, b2m +/+ offspring of poly(I:C) injected b2m +/- mothers do not exhibit the expected behavioral abnormalities in open field exploration or stereotyped marble burying behavior. That is, there is no significant difference in behavioral performance between b2m+/+ poly(I:C) versus saline offspring (Figure 3 and 4). One possibility underlying this result may be discrepancies in the degree of immune activation experienced by b2m +/- mice in response to poly(I:C). Future studies evaluating MIA-induced cytokine levels in the maternal sera in b2m+/- versus WT controls will provide insight into any potential differences in intensity of MIA experienced by the different strains of mice.

Discussion

That the experimental condition representative of our positive control (b2m+/+ offspring of b2m +/- mothers) do not exhibit expected abnormalities in open field exploration and marble burying after maternal poly(I:C) injection suggests disparity between the b2m+/- maternal response to poly(I:C) compared to that experienced by wildtype b2m+/+ controls. This confounding factor precludes the ability to use this transgenic line to evaluate gene x environmental interactions in the development of autism and schizophrenia-related behavior. Future studies assessing the poly(I:C)-induced antiviral response in b2m +/- versus wildtype mice should be performed; specifically, levels of the cytokines IL-6, IL-1b and TNFa can be measured in maternal sera in poly(I:C)-injected b2m+/- and wildtype mice as an indication of poy(I:C)-induced immune response.

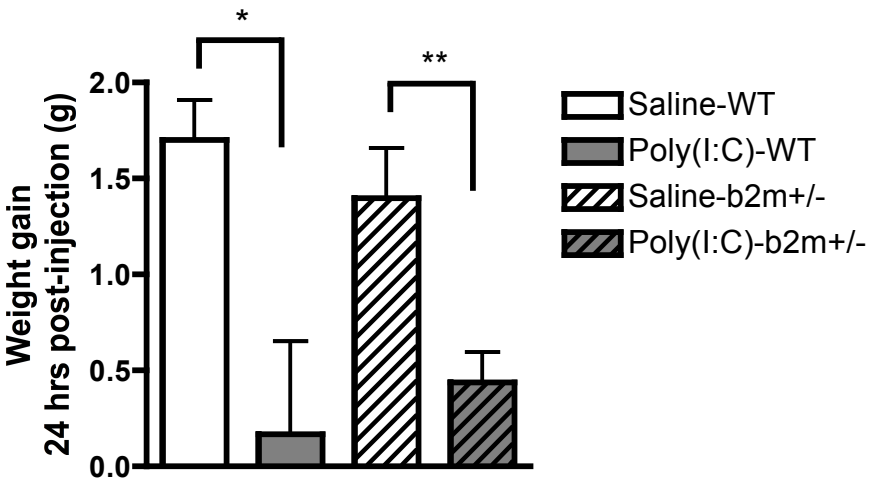

Figure 1. Heterozygous b2m mice exhibit expected maternal weight loss after poly(I:C) injection. Maternal poly(I:C) injection into wildtype mice leads to transient weight loss seen at 24 hours post injection, compared to saline-injected controls. Heterozygous b2m females mated with heterozygous b2m males display a comparable decrease in weight after poly(I:C) injection.

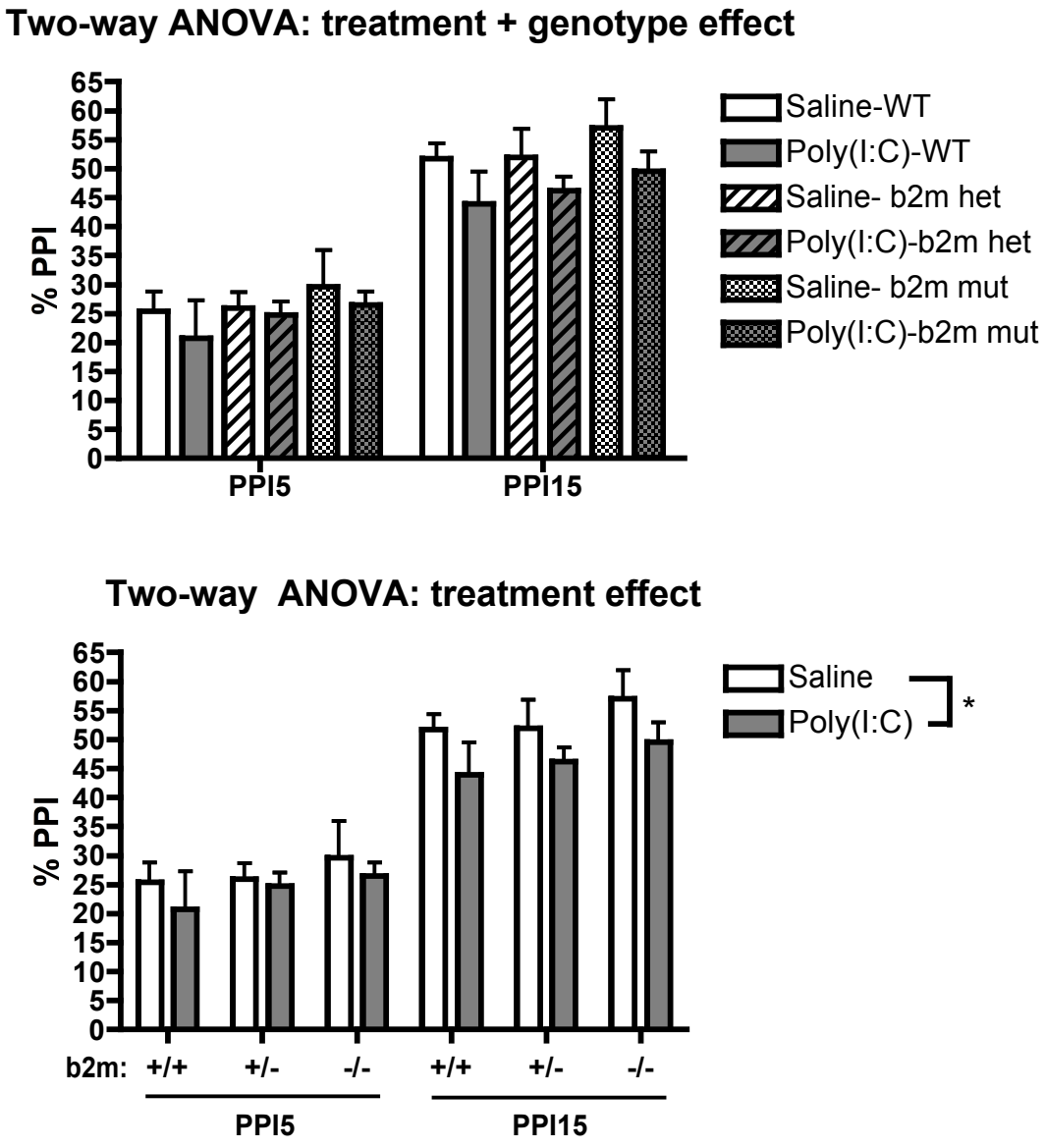

Figure 2: There is no effect of b2m genotype on MIA-induced deficits in PPI. B2m +/+, +/- and -/- offspring display no difference in percent PPI after 5 or 15 db prepulse. However, maternal poly(I:C) injection into pregnant b2m +/- mice induces a significant treatment effect of lowering PPI, consistent with that observed after MIA in WT mice.

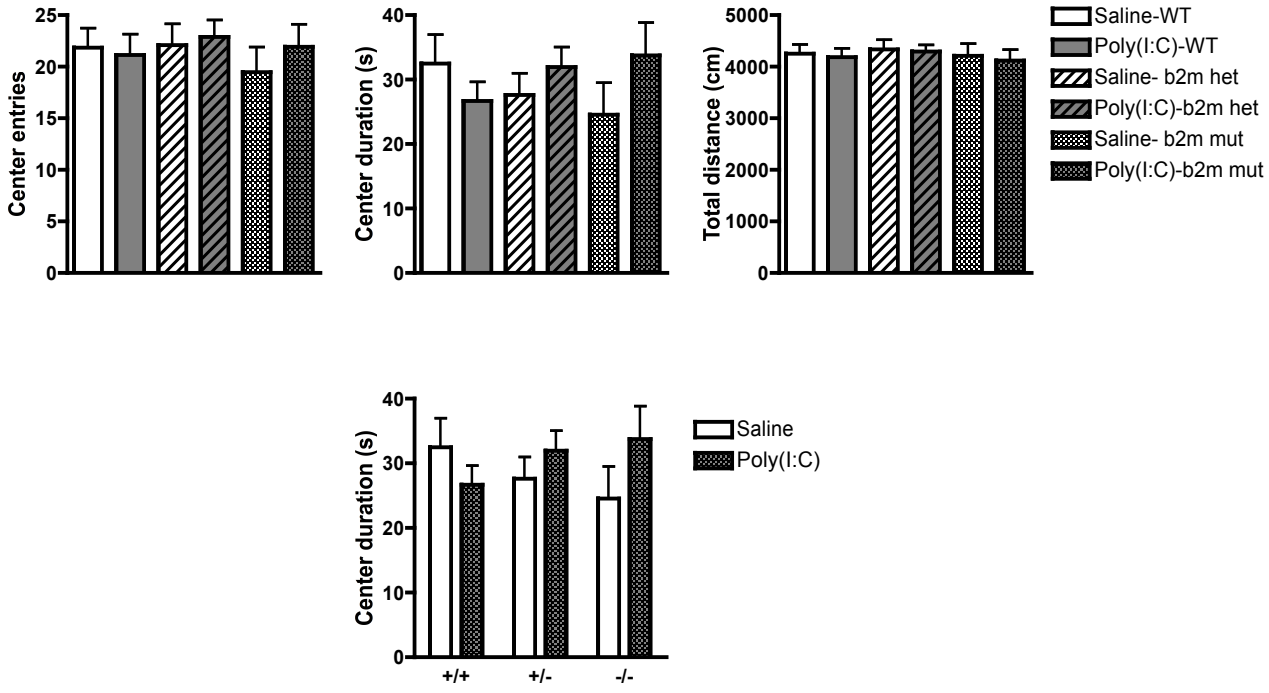

Figure 3. There is no effect of MIA treatment or b2m genotype on open field exploration. B2m+/+ offspring of MIA versus control mothers exhibit no difference in open field performance as measured by center entries or center duration into the center of the open field. This is in contrast to the expected effect of MIA on inducing anxiety-like behavior in the open field.

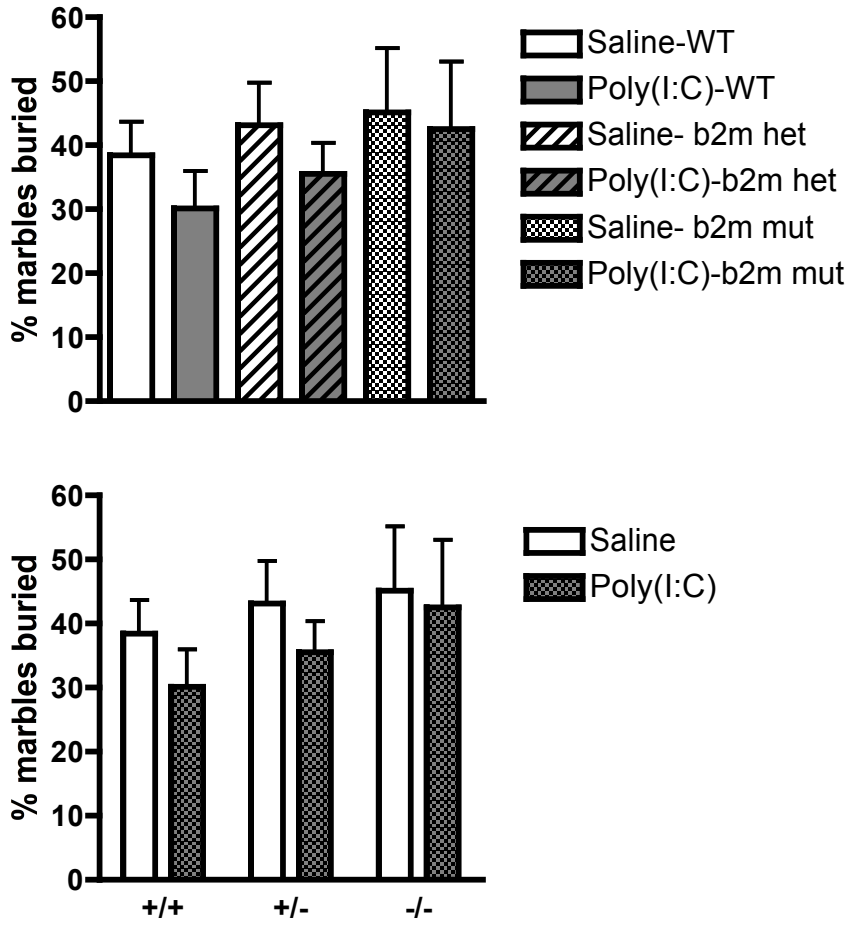

Figure 4. There is no effect of MIA treatment or b2m genotype on stereotyped marble burying behavior. B2m+/+ offspring of MIA versus control mothers exhibit no difference in stereotyped marble burying. This is in contrast to the expected effect of MIA on inducing repetitive behavior in the marble burying task.