MATERNAL IMMUNE ACTIVATION AND ABNORMAL BEHAVIOR IN THE ADULT OFFSPRING: TOWARDS A MECHANISM

Thesis by

Stephen Edward Paucha Smith

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Abstract

Maternal infection is a risk factor for both schizophrenia and autism. The offspring of women who develop an infection during pregnancy are several times more likely to develop these diseases compared to offspring from uncomplicated pregnancies. Modeling this risk factor in animals, when pregnant rodents are given an influenza infection during pregnancy, their offspring show several behavioral and histological abnormalities consistent with human mental illness. Maternal injection of non-infectious, immune-activating compounds, such as the dsRNA poly(I:C), yields similar results, suggesting that the maternal immune response causes deleterious changes in fetal brain development. The main focus of this thesis is establishing the importance of a single component of the maternal immune response, the cytokine interleukin-6 (IL-6), in mediating the observed changes in the brain development and behavior of the offspring. In addition, I report new observations on the offspring of poly(I:C)-activated pregnant mice that are consistent with findings in autism and schizophrenia: a localized deficit of Purkinje cells in the cerebellum, abnormal eye-blink conditioning, abnormal hippocampal-dependent behaviors and hyper-sensitivity to dopamine in the hippocampus. I also present data on the immune reaction to poly(I:C) in pregnant non-human primates. Finally, I describe preliminary findings on the identification of factors that act down-stream of IL-6. The mechanism through which maternal immune activation causes abnormal behavior in the offspring could illuminate important pathways that contribute to the pathogenesis of schizophrenia and autism.

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Chapter 1

Summary Chapter

Stephen E. P. Smith

Maternal infection is a risk factor for both schizophrenia and autism, and the evidence indicates that the maternal immune system, rather than direct infection of the fetus, is what alters fetal brain development. While an excellent animal model of maternal influenza infection has been established, for several reasons it is desirable to use an alternate approach to activate the maternal immune system. For example, it is difficult to give identical respiratory infections to a cohort of animals because two mice given an identical load of virus can display very different sickness behavior, cytokine levels and viral load. Also, manipulations of the immune system during viral infection can increase the severity of disease due to the necessity of a functioning immune system to fight infection. By using the synthetic double-stranded RNA, poly(I:C) to induce maternal immune activation (MIA), we are able to study the effects of MIA on the embryo, and manipulate the maternal immune system, while avoiding the problems raised above.

The majority of my thesis work has focused on this model of MIA, in which a pregnant mouse is injected with poly(I:C) on day 12 of pregnancy. This simple manipulation causes many behavioral, histological and gene expression changes in the adult offspring that are relevant to human schizophrenia and autism. The following chapters report our investigations of the mechanism through which MIA causes long-lasting changes in the fetal brain, and characterization of the nature of these changes. The overall goal of this work is to better understand etiology, pathogenesis and potential interventions for the devastating diseases of schizophrenia and autism.

Chapter II is a literature review that has been submitted for publication in the book *The Neuroimmunological Basis of Behavior and Mental Disorders,* scheduled for publication in 2008. I wrote the review and created the tables and figure, with advice

from Dr. Paul Patterson, the senior author. This chapter discusses the human evidence that suggests maternal infection is a risk factor for both schizophrenia and autism, and the animal models that attempt to replicate this observation in the laboratory. Finally, the chapter ends with a speculative discussion on possible mechanisms through which MIA leads to abnormal behavior in the offspring.

Chapter III appeared in the *Journal of Neuroscience* in October, 2007. This paper reports the observation that the cytokine interleukin-6 (IL-6) is central to the mechanism through which MIA causes long-lasting changes in the adult offspring. I am first author of this chapter, and I performed the majority of the experiments and wrote the first draft of the manuscript. Jennifer Li, the second author, was an undergraduate student in the Patterson laboratory from 2002 to 2004 who performed the early pilot experiments injecting cytokines into pregnant mice and observing the behavior of the adult offspring. Dr. Karoly Mirnics and his postdoctoral fellow, Dr. Kassimira Garbett, the third and fourth authors, preformed the microarray experiments using tissue that I collected. They have published on transcriptional changes in human schizophrenia and autism, as well as several other disorders.

Chapter IV has been submitted for publication. This paper reports the observation that the offspring of both maternal flu- and poly(I:C)-treated animals display a decreased linear density of Purkinje cells in the cerebellum, which resembles the cerebellar pathology seen in schizophrenia, and particularly in autism. I am the second author of this chapter behind Dr. Limin Shi. Dr Shi performed the maternal infection experiments and wrote the first draft of the manuscript. I performed the maternal poly(I:C) experiments, including histological analysis, and made major revisions to the manuscript.

The third and fourth authors, postdoctoral fellow Dr. Natalia Malkova and undergraduate student Doris Tse, assisted Dr. Shi in her experiments.

Chapter V represents a collaboration between Dr. Ka-Hung Lee, a postdoctoral fellow in Dr. Richard Thompson's laboratory at the University of Southern California, and myself. Dr. Lee is an expert in eye-blink conditioning in mice, and performed the electrode implantation and the testing on mice that I generated at U.S.C. I wrote the chapter for inclusion in this thesis, and as more data is collected we plan to submit a manuscript for peer-reviewed publication with Dr. Lee as the first author. This chapter reports observations on abnormal eye-blink conditioning in the adult offspring of poly(I:C)-treated mothers. This behavior is relevant because of the eyeblink conditioning abnormalities that have been reported in both schizophrenia and autism.

Chapter VI represents a collaboration between Hiroshi Ito, a graduate student in Erin Schumann's lab at Caltech, and myself. Hiroshi Ito is an expert electrophysiologist who specializes in studies of the effects of dopamine on hippocampal slices. Because of the known abnormalities in the dopaminergic systems of both schizophrenic patients and our poly(I:C)-exposed mice, I hypothesized that Hiroshi might be able to identify electrophysiological abnormalities in the mice. I provided him with behaviorally characterized mice, and he performed electrophsiological tests on them. I wrote the chapter for inclusion in this thesis and as more data is collected we plan to submit a manuscript for peer-reviewed publication with Hiroshi Ito as the first author.

Finally, the appendices present research that is too preliminary for inclusion in the main chapters, but that adds important observations about the MIA model. Appendix A

reports recent attempts by myself, a lab volunteer, Hae Jin Kang, and a Caltech undergraduate, Jennifer Li, to establish a model of MIA that displays brain inflammation like that seen in autism. This work is ongoing. Appendix B covers work by myself, with a contribution by rotating graduate student Illana Goldflam, on the effects of MIA on the placenta. Appendix C describes work towards the establishment of a model of gene-environment interaction using a DISC-1 mutant mouse. This work is being continued by a postdoctoral fellow in our lab, Catherine Bregere, and will eventually be included in a publication with a Canadian group working with different strains of DISC-1 mice. Appendix D describes cytokine assays that I performed as part of a collaboration with U.C. Davis, in which we aim to establish the MIA model in a non-human primate. The offspring of these monkeys will be behaviorally analyzed by the group of Dr. David Amaral at U.C. Davis. Appendix E describes preliminary results looking for targets downstream of IL-6 in the fetal brain, in an attempt to extend the mechanism of maternal immune activation beyond the cytokine. Appendix F describes a method that I developed for maintaining physiologically relevant levels of cytokines in the serum of mice without continued stress from injections or minipumps by electroporating plasmid DNA in the leg muscles. This technique may be useful in the future. Finally, Appendix G describes a collaboration with the Gage laboratory at the Salk Institute, analyzing retrotransposon activation during MIA in L1 transgenic mice. I generated the MIA offspring and postdoctoral fellow Dr. Alysson Muotri preformed the majority of the described experiments. If the preliminary data are replicated, this would also lead to a publication.

Chapter 2

Maternal Immune Challenge Alters Neurodevelopment and Behavior in the Adult Offspring

Stephen E.P. Smith and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA 91125

Written by Stephen Smith for inclusion in the forthcoming book *The Neuroimmunological Basis of Behavior and Mental Disorders*, winter 2007/8.

The immune system rapidly responds to pathogens by releasing a variety of signaling molecules that trigger a number of infection-fighting cellular programs. These same signaling pathways (e.g. NF-kB, JAX/STAT, ERK) are used by the developing brain to orchestrate programs of cell proliferation, differentiation and migration. Thus, when a pregnant woman falls ill, there is the potential for cross-talk between the maternal immune response and the developing fetal brain. In fact, maternal infections are significant environmental risk factors for schizophrenia and autism. There are several animal models in which infection-induced maternal immune activation causes behavioral, histological and gene expression changes in the offspring that are reminiscent of human mental disorders. We review both human and animal data that demonstrate these effects of maternal immune activation, and discuss potential mechanisms through which the maternal immune system may alter brain development.

Genes vs. environment in mental disease

It is well known that genes play a major role in several mental disorders. However, in our view, the genetic contributions to schizophrenia and autism, in particular, can be over-emphasized. While genes undeniably play a major role, only 5-10% of autism cases can be can be attributed to known chromosomal abnormalities or single gene mutations. Furthermore, while early estimates of monozygotic twin concordance rates in autism were as high as 90%, recent reports put that number closer to 60% (Lemery-Chalfant et al., 2006). Similarly, with notable exceptions (e.g. DISC1), very few cases of schizophrenia can be traced to a known genetic cause. While this

discrepancy may be attributed to the complexity of studying diseases where multiple genes each contribute small amounts to risk, the concordance rate for monozygotic twins in schizophrenia (50%) leaves much room for environmental factors. Moreover, indirect evidence reveals an important finding: monozygotic twins that share a placenta have a high concordance rate (60%) for schizophrenia, while those with separate placentas have a concordance rate similar to that of dizygotic twins (Davis et al., 1995; Phelps et al., 1997). In addition, the concordance rate of dizygotic twins (~17%) is almost twice as high as that of siblings (~9%), even though these groups have identical genetic relatedness. These findings highlight the importance of the intrauterine environment, which is further emphasized by human and animal studies of maternal infection.

Prenatal infections and mental disorders

Maternal infection by several different organisms during early- to mid-pregnancy has been linked to both schizophrenia and autism. The strongest evidence for maternal infection increasing risk for a mental disorder in the offspring is the connection between schizophrenia and maternal respiratory infection. In a pioneering study, Mednick et al. (1988) found a higher rate of schizophrenia among a cohort of Swedish adults who were *in utero* during the 1957 influenza epidemic. Since then, over 25 epidemiological studies have assessed the rate of schizophrenia in people who were *in utero* during influenza epidemics, and the majority have found an increased incidence of disease among the exposed offspring (reviewed by Bagalkote et al. (2001)). However, this epidemiological data is population-based and therefore is unable to document a direct relationship between respiratory infection in individual mothers and later development of schizophrenia in the offspring. While this caveat should decrease the probability of finding an association, it

nevertheless creates uncertainty in the conclusions. Brown, Susser and colleagues were able to overcome this limitation by using a large pool of banked maternal serum samples that were linked to detailed medical records of both mothers and offspring (Brown et al., 2004a). They found that in cases where they were able to confirm maternal influenza infection by antibody assays of the banked serum, the resulting offspring were 3-7 times more likely to develop schizophrenia as adults. Due to the high prevalence of influenza, they estimate that 14-21% of the schizophrenia cases would not have occurred if maternal infection had been prevented. The same group has also found associations between schizophrenia in the offspring and maternal toxoplasmosis (Brown et al., 2005), genital/reproductive viral infection (Babulas et al., 2006) or elevated levels of maternal interleukin-8 (Brown et al., 2004b). Remarkably, this association was detected despite the inability to screen for a susceptibility genotype. If one assumes that only genetically susceptible individuals will develop schizophrenia after maternal infection, the increased risk due to infection will be much higher than 3-7-fold in this group.

Although there is much less evidence available, a link has also been found between maternal infection and autism in the offspring. Rubella epidemics in the 1960s were associated with greatly increased risk for autism in children that were exposed *in utero*, as well as for physical abnormalities and mental retardation (Desmond et al., 1967; Chess, 1977). Since the development of the rubella vaccine, many fewer cases of maternal rubella infection are seen. Small studies of other maternal infections, such as toxoplasma, syphilis, varicella and rubeola also support the idea that maternal infection can be a risk factor for autism (Ciaranello and Ciaranello, 1995; Hyman et al., 2005). While the phenotypic heterogeneity and complex genetics of schizophrenia and autism

make it difficult to establish maternal infection as a definitive cause of the disorders, there is considerable evidence implicating it as an important risk factor.

Animal models of immune activation

The diversity of infections that have been implicated as risk factors for mental disorders, as well the fact that many of these infections do not have direct access to the fetal compartment, has led to the hypothesis that the maternal immune system, rather than a specific pathogen, is responsible for the increased incidence of mental disease in the offspring (Patterson, 2002, 2005). While this hypothesis is not testable in humans, animal models have shown that maternal immune activation is able to cause a variety of behavioral, histological and transcriptional changes in the adult offspring. The models use one of three methods to induce maternal immune activation in pregnant rodents: influenza infection, injection of the synthetic double-stranded RNA, poly(I:C), or injection of bacterial lipopolysaccharide (LPS). Neuropathology, gene expression profiling, electrophysiology, behavioral assays and antipsychotic drug treatment demonstrate similarities between the "exposed" offspring and humans suffering from mental disease. Unfortunately, it is often difficult to directly compare the results from different research groups, as variables such as the species used, the compound administered, dosing, timing of and method of treatment, and tests of outcome are often different among laboratories. Taken together, however, these models strongly support the hypothesis that maternal immune activation can have deleterious effects on the offspring *in utero* (see Table I).

Assaying features of mental illness in mice

Before discussing the specific rodent models, it will be useful to briefly review some of the more common behavioral tests that are used to model schizophrenic- and autistic-like symptoms in mice. Schizophrenia and autism are both assessed using the diagnostics and statistical manual, now in its fourth volume (DSM-IV). The diagnosis involves an interview, and is based upon behaviors, some of which are difficult to model in animals (e.g. disordered thoughts or language delay). Fortunately, there are several endophenotypes, traits that are not diagnostic but are found at a greater frequency in populations of affected individuals, which can be measured in experimental animals.

Pre-pulse inhibition (PPI) is a measure of sensory-motor gating that is disrupted in schizophrenic (Turetsky et al., 2007) and autistic (Perry et al., 2006) individuals, as well as in people who have other mental health problems. High-functioning autistics describe being bombarded with an overwhelming amount of sensory information, and deficits in PPI may reflect an underlying inability to quickly classify sensory input as relevant or irrelevant. PPI refers to the inhibition of a startle response to an aversive stimulus (a "pulse") when the startling stimulus is preceded by a smaller, non-startling stimulus (a "pre-pulse"). The interval between the pulse and prepulse ranges from 50 to 500 ms, which does not allow time for higher-level processing of the two stimuli. In rodents, PPI is assayed by placing the animal in a small enclosure and measuring its startle response to a loud pulse of white noise; in humans, the stimuli are usually airpuffs to the eye.

Latent inhibition (LI) is another behavioral test that measures the ability of a subject to ignore or "filter out" irrelevant information, and is highly pertinent to schizophrenia

(Weiner, 2003). The neural circuitry for LI lies in the hippocampus and nucleus accumbens, and relies heavily on dopaminergic transmission between these areas. LI is disrupted in schizophrenic subjects and in amphetamine-treated humans and rats, is restored to normal levels in schizophrenics by anti-psychotic drugs, and is enhanced in normal humans and rats by these drugs (Weiner, 2003). Measuring LI involves repeatedly exposing the subject to a conditioned stimulus (CS), and then pairing that stimulus with a different, unconditioned stimulus (US). Pre-exposed (PE) animals will not associate the CS and the US as strongly as non-pre-exposed (NPE) animals; the difference between the responses of PE and NPE animals is termed LI. Both PPI and LI tests are easily administered to both humans and experimental animals, making them ideal tools for the validation of animal models.

Several other tests have been developed in animals to model features of human mental disorders. Heightened fear and anxiety are features of many mental diseases, and the open field test, which involves placing a mouse in a brightly-lit enclosure and monitoring its movement, measures anxiety levels in rodents. Fewer entries into the center of the field and shorter distances moved in exploration are relevant measures. Interestingly, studies of the exploratory behavior of autistic children have validated the rodent paradigm by showing reduced exploration of a novel object-filled room by affected children (Pierce and Courchesne, 2001; Akshoomoff et al., 2004). Finally, social interaction is a central deficit in autism, and several tests have been developed to monitor the interaction between two mice placed in a shared enclosure (Crawley, 2007).

Maternal influenza infection

The influenza infection model is based directly on human data showing a higher incidence of schizophrenia in offspring of mothers who developed influenza infections during the second trimester of pregnancy (Fatemi et al., 2002; Shi et al., 2003). Mice are inoculated intra-nasally with a mouse-adapted human influenza virus on day 9.5 of pregnancy. Over about seven days, the mice develop fluid in the lungs, show noticeable sickness behavior, and have elevated serum levels of several cytokines. As mouse pregnancy lasts 18-19 days, the sickness persists for the second half of mouse pregnancy. Due to differences in mouse vs. human fetal development, namely that mice are born with their brains in a less mature state than humans, this time period corresponds to the human 2nd trimester in terms of brain development milestones (for an excellent review of inter-species developmental stages, see (Clancy et al., 2001)). Thus, this model closely recapitulates the human risk factor of a second-trimester influenza infection.

The adult offspring of influenza-infected mice appear superficially normal, but display several behavior abnormalities that are highly relevant to schizophrenia and autism. They have lower PPI than controls, and this deficit is rescued by acute treatment with antipsychotic drugs. They display heightened anxiety in the open field, as measured by a reduced total distance moved, less rearing, and fewer entries into the center of the field. Finally, they show less social interaction with an unfamiliar, same-sex conspecific (Shi et al., 2003). Offspring of flu-infected mothers also display several histological abnormalities that are reminiscent of those found in mental disorders. For example, the most commonly reported histological finding in post-mortem autistic brains is a selective loss of Purkinje cells in lobules VI and VII of the cerebellum, and structural MRI studies have also found reduced volume of autistic cerebella (Palmen et al., 2004). Remarkably, offspring of the

influenza-exposed mice also show a highly selective reduction in the linear density of PCs in lobules VI and VII of the cerebellum, a deficit that seems to be of developmental origin (Shi et al., Submitted). Other relevant histological findings include altered levels of synaptosome-associated protein-25 (SNAP-25), reduced reelin immunoreactivity in the cortex, and smaller, more densely packed pyramidal cells in the hippocampus (Fatemi et al., 1998; Fatemi et al., 1999; Fatemi et al., 2002).

Maternal Immune Activation

Several lines of evidence suggest that maternal immune activation (MIA), rather than direct infection of the fetus, is responsible for the behavioral and histological changes seen the offspring of infected mothers. First, in human studies, the fact that a wide variety of pathogens have similar effects suggests that they act via a similar mechanism. Furthermore, many of the implicated infections are confined to specific areas of the body, and do not involve the fetus. For example, influenza infection is typically confined to the respiratory system. This was confirmed in the influenza mouse model when using a sensitive RT-PCR assay that can detect as little as one plaque-forming unit of virus; no virus was detected in the exposed fetuses (Shi et al., 2005). Finally, and most convincingly, 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 pathogens.

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 (TLR3). Double-stranded RNA is a sign of viral infection for the innate

immune system; activation of TLR3 induces an inflammatory cascade that results in the production of anti-viral cytokines and chemokines. Injection of poly(I:C) in a 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, LI, open field exploration and social interaction (Shi et al., 2003; Zuckerman et al., 2003; Zuckerman and Weiner, 2005; Smith et al., 2007). Many of these behavioral deficits respond to antipsychotic drugs (Zuckerman et al., 2003; Zuckerman and Weiner, 2005; Ozawa et al., 2006). Furthermore, the PPI and LI deficits only occur in post-puberty, mimicking the adult-onset of schizophrenia (Zuckerman et al., 2003; Ozawa et al., 2006). Poly(I:C) also causes enlarged ventricals (Piontkewitz et al., 2007) and altered dopamine metabolism in the adult offspring (Ozawa et al., 2006), which are relevant to schizophrenia, and increased $GABA_A$ receptor expression in the adult offspring (Nyffeler et al., 2006), which is also relevant for autism. Also in common with the influenza model, maternal poly(I:C) treatment causes a deficit of Purkinje cells in lobule VII of the cerebellum (Shi et al., submitted).

Maternal LPS administration

Another method of inducing MIA is the injection of bacterial lipopolysaccharide (LPS), a natural ligand for the toll-like receptor-4 (TLR4). Intrauterine bacterial infection is commonly associated with preterm birth, neurological disorders such as cerebral palsy, and mental retardation (Saliba and Henrot, 2001; Dammann et al., 2002). Intrauterine infection leads to pathology, such as white-matter damage, which is more severe than that found in schizophrenia and autism. Very limited evidence links maternal bacterial infection to the later development of schizophrenia (Watson et al., 1984; O'Callaghan et

al., 1994). However, activation of TLR4 activates many of the same signaling pathways as TLR3, and elevates levels of many of the same cytokines in the maternal circulation, notably IL-6. The specific combination of cytokines and chemokines is different than for TLR3, but like poly(I:C), LPS produces a very strong, but transient, immune activation. Many of the behavioral abnormalities seen in the offspring of poly(I:C)-treated mothers are also seen in the offspring of LPS-treated mothers. Using a very severe protocol of LPS injections daily throughout pregnancy, one group has reported PPI deficits (Borrell et al., 2002) that are corrected by administration of antipsychotic drugs (Romero et al., 2007) in adult offspring. However, two injections of 50ug/kg LPS in late pregnancy (E18 and 19) does not yield PPI deficits (Fortier et al., 2004). Increased anxiety-like behavior and abnormal social behavior (Hava et al., 2006), as well as enhanced amphetamine-induced locomotion (Fortier et al., 2004) and abnormal learning and memory (Golan et al., 2005) have been reported in the offspring of mice given LPS injections on E17, 18 or 19**.** Histological findings include fewer, more densely packed neurons in the hippocampus (Golan et al., 2005), increased microglial staining (Cai et al., 2000; Borrell et al., 2002; Ling et al., 2004; Paintlia et al., 2004), increased glial fibrillary acidic protein (GFAP) staining (Cai et al., 2000; Borrell et al., 2002), altered tyrosine hydroxylase (TH) staining (Borrell et al., 2002; Ling et al., 2004) and decreased myelin basic protein (MBP) staining (Cai et al., 2000; Paintlia et al., 2004), all potentially relevant for mental illness.

Other relevant animal models of environmental risk factors

Several other protocols for inducing brain pathology and behavioral abnormalities in pre- or early postnatal animals exist, including intrauterine infection with periodontal

bacteria (Lin et al., 2003a; Lin et al., 2003b; Han et al., 2004; Bobetsis et al., 2006), injection of LPS directly into the fetus, (reviewed by Wang et al. (2006)) or injection of cytokines in early postnatal animals (reviewed by Nawa and Takei (2006)). However, as these methods are not meant to model a second-trimester maternal infection, and are not known to be directly relevant to schizophrenia and autism, they are not reviewed here. There is also a significant body of research on maternal stress as a risk factor for schizophrenia (reviewed by (Relier, 2001)), and in rodents, maternal behavioral stress leads to abnormal behavior in the adult offspring, although the assays used are different (reviewed by Weinstock (2001)).

Mechanisms of behavioral abnormalities caused by MIA

The mechanisms and pathways by which MIA alters behavior and fetal brain development is beginning to be explored, with particular focus on cytokines. Cytokines are small (8-30 kDa) signaling molecules that are released in response to a wide range of immune challenges. They are attractive candidates for causing the observed changes in fetal brain development for several reasons. First, accessibility: they are released into the maternal serum in response to infection/MIA; thus, even though an influenza infection is confined to the lungs, cytokines produced at the infection site will have access to the fetus. Moreover, evidence indicates that cytokines can cross the placenta and access the fetus (Dahlgren et al., 2006; Ponzio et al., 2007). Second, activity: Cytokines signal through several key developmental pathways, including the STAT, NF-kB, and ERK cascades, allowing for the possibility of interference with those signaling pathways. Moreover, many cytokine receptors are expressed in both developing and mature neurons and glia, and when activated, can cause morphological and functional changes in those

cells (Jankowsky and Patterson, 1999; Gilmore et al., 2004; Bauer et al., 2007). Thus, cytokines are logical candidates to perturb fetal brain development.

Altered serum cytokine levels in response to MIA have been documented in several of the animal models discussed above. Consistently, regardless of the method employed, the pro-inflammatory cytokines IL-6, IL-1β and TNFα are elevated in the maternal serum and placenta. Our group has identified at least 10 more cytokines and chemokines that are upregulated in maternal serum after poly(I:C) administration. At least some of these cytokines are able to cross the placenta and gain access to the fetal brain (see Table II). Radiolabeled IL-6 and IL-2 have both been found to cross the placenta; when injected i.v. in pregnant rodents, radioactivity levels in the fetuses are 15-20% of those in maternal serum (Dahlgren et al., 2006; Ponzio et al., 2007). Whether cytokines actually cross into the fetal brain during experimentally-induced MIA is a matter of contention. Some groups have reported that IL-6 protein is significantly elevated in the fetal brain following MIA (Meyer, 2006), although negative or inconclusive results have also been reported (Meyer 2007, Ashdown, 2006). Similar mixed results have been reported for TNFα; reports have shown a small increase (Urakubo et al., 2001), a significant increase (Bell et al., 2004) or undetectable (Ashdown et al., 2006) levels of TNAα protein or mRNA in fetal brains after MIA. The different cytokine responses in the fetal brain are likely due to the different severity of the treatment administered in different laboratories. For example, induction of severe inflammation by injecting LPS directly into the uterine horn produces large increases in the levels of cytokine mRNAs in the fetal brain (Elovitz et al., 2006). Cytokine mRNA increases in fetal brain have also been reported following i.v. injection of poly(I:C)

(Meyer et al., 2006b; Meyer et al., 2007). Our group has administered a relatively mild dose of poly(I:C) (single i.p. injection), and we observe only small, nonsignificant changes in the levels of cytokine proteins in the fetal brain (W. Xu, B. Deverman, S. Smith, unpublished data). However, since the cytokine levels under discussion approach the lower detection limit of the assays, some of the negative results may reflect limitations of the assays rather than a lack of cytokine access to the brain. The radiolabeled cytokine experiments, as well as preliminary data from our group showing increased mRNA of downstream genes in fetal brains of poly(I:C)-treated mothers (E. Hsaio, S. Smith, unpublished data), suggest that cytokines are able to cross the placenta and gain access to the fetal brain, despite remaining undetectable by standard ELISA assays. Since there are also reports of cytokine mRNA induction in the fetal brain, it is also possible that other signaling mechanisms (fever, ischemia, nutritional changes) could induce cytokines in the fetus directly, or indirectly, by altering the placenta.

Recent work has shown that the cytokine IL-6 plays a critical role in the manifestation of behavioral deficits caused by MIA. Samuelsson et al. (2006) administered three i.p. injections of IL-6 to pregnant rats over the course of six days. They found a working memory deficit in the adult offspring, as well as elevated IL-6 levels in the adult hippocampus, indicating an ongoing inflammation triggered by early events. This inflammatory state is reminiscent of the profound inflammation in autistic brains (Vargas et al., 2005), in which IL-6 was among the most prominently up-regulated cytokines in subjects ranging in age from 5-44. Moreover, the IL-6-exposed adult rat offspring had elevated GFAP and $GABA_A$ receptor levels, similar to those reported in some MIA offspring (Nyffeler et al., 2006).

We have also studied the effects of both raising IL-6 levels in pregnant mice and blocking endogenous IL-6 in the poly(I:C) MIA model (Smith et al., 2007). In pregnant mice injected once with 5 mg of IL-6, we found both PPI and LI deficits in the adult offspring. Other injected cytokines (IL-1α, TNFα and IFNγ) had no effect on the behavior of the adult offspring. We used neutralizing antibodies to selectively block cytokines during poly(I:C)-induced MIA; co-administration of an anti-IL6 antibody completely prevented all of the behavioral deficits induced by poly(I:C) (deficits in PPI, LI, and social interaction, as well as increased open field anxiety). In contrast, neutralization of IL-1β or IFNγ did not rescue the poly(I:C)-induced behavioral deficits. Moreover, IL-6 knockout (KO) mice are insensitive to the effects of MIA; offspring of pregnant IL-6 KO mice that were treated with poly(I:C) do not display PPI or LI deficits. We also used a microarray analysis to monitor changes in the adult brain transcriptome caused by MIA. Of 61 genes whose expression was altered by MIA, 55 were normalized in the offspring of pregnant mice that were co-injected with poly(I:C) and anti-IL-6. Thus, blocking IL-6 prevents the changes in behavior and gene expression caused by MIA (Smith et al., 2007).

Based on these results, an attractive, but preliminary, hypothesis can be proposed for the mechanism of MIA-induced behavioral deficits (Fig. 1). Maternal immune activation induces production of cytokines, particularly IL-6, which enter the maternal circulation. In mid-, but not late gestation, IL-6 crosses into the fetal circulation (Dahlgren et al., 2006), which correlates with human epidemiological data suggesting that early, not late, infections increase risk for mental disorders (Brown et al., 2004a). IL-6 can have variety of direct effects on the developing brain (reviewed by Bauer et al.,

2007). Recent studies in our group indicate that offspring of influenza-infected mice have abnormal neuron migration to cortical layers II/III (Limin Shi, personal communication) as well as fewer Purkinje cells in lobules VI and VII of the cerebellum (Shi et al., submitted). In addition, IL-6 causes neurons in tissue culture to retract their processes, which suggests that early morphological changes in the developing brain could precipitate future behavioral abnormalities (Gilmore et al., 2004). Finally, the STAT-3 pathway, through which IL-6 signals, regulates developmental processes such as the switch between neurogenesis and gliogenesis (Bauer et al., 2007). The potential for IL-6 to alter fetal development indirectly by changing placental properties such as vascularization (Paul et al., 2003), or by breaking down maternal immune tolerance of the fetus (Sargent et al., 2006), should also be considered.

Implications and potential therapies

One important implication of the demonstration of the key role of IL-6 in the effects of MIA is that it suggests prevention or treatments based on anti-cytokine or anti-inflammatory therapies. There are two potential time-points for intervention: at the time of the maternal infection, and post-partum, when the behavioral deficits have already manifested. Potential interventions at the time of infection are complicated by the need to fight the infection. Although blocking IL-6 prevents the deficits caused by poly(I:C), if IL-6 is blocked during an influenza infection in a pregnant mouse, the mouse suffers miscarriage and will often succumb to the illness (S. Smith, unpublished). A similar effect is observed in IL-6 KO mice, indicating that IL-6 is necessary to fight infection. Thus, direct neutralization of IL-6 is not a viable clinical option. Other treatments that reduce the inflammatory response, but still allow effective control of infection may be

possible. Anti-inflammatory treatment with N-acetylcysteine suppresses the fetal inflammatory response after maternal LPS administration (Beloosesky et al., 2006), but it remains to be seen if this treatment would be viable in an infection model. The anti-inflammatory cytokine IL-10 is naturally increased during normal pregnancy, and endogenous IL-10 is essential for resistance to LPS-induced pregnancy loss and pre-term labor in mice (Robertson et al., 2006). Recently, Meyer et al. (2007) showed that macrophage-specific overexpression of IL-10 prevents behavioral deficits in the offspring that are caused by maternal poly(I:C) administration. The behavioral deficits may be prevented by the IL-10-induced reduction in the concentration of IL-6 and TNFα in maternal serum after poly(I:C) injection. One caveat of this work is that the genotype of the offspring was not addressed, so the behavioral results may have stemmed from a post-natal, anti-inflammatory action of IL-10 overexpression, which would be the equivalent of treatments discussed below. Further, the observation that IL-10 overexpression in the absence of poly(I:C) treatment induces behavior abnormalities in the offspring (Meyer et al., 2007) highlights the inherent dangers of prenatal interventions. Without a way to predict which offspring might be susceptible to develop schizophrenia or autism when exposed to MIA, it is unlikely that the FDA would approve clinical trials for these types of early interventions.

It is also possible that MIA sets in motion an ongoing immune activation or dysregulation in the brain that may be responsible for some of the behavioral abnormalities observed in the adult offspring. Both schizophrenic (Garver et al., 2003; Zhang et al., 2005) and autistic (Singh et al., 1991; Croonenberghs et al., 2002; Zimmerman et al., 2005) subjects show signs of abnormal peripheral immune systems,

with reports of elevated cytokines in blood. Recent microarray data show dysregulation of immune-related transcripts in both schizophrenic (Arion et al., 2007; Saetre et al., 2007) and autistic (Garbett et al., 2007) brains. A 50-fold increase in TNFα levels was found in a study of CSF from ten autistic patients (Chez et al., 2007). Moreover, there is severe inflammation in the brains of autistic patients, from a broad range of ages (5 - 44 yrs) and heterogeneity in diagnoses (regressive vs. non-regressive, epilepsy, retardation) (Vargas et al., 2005). Many cytokines and chemokines are elevated in tissue from both the cortex and the cerebellum, and a high density of activated microglia and astrocytes are present, indicating an active cellular inflammatory process. A replication of this work was recently presented, showing increased Iba-1 microglial immunoreactivity in 6 autistic subjects compared to age-matched controls (Morgan et al., 2007). It is likely that this chronic elevation of cytokines and associated cellular inflammation would have an adverse, acute effect on the behavior of the patients, perhaps even causing some of the core features of the disorders. It is clear, for instance, that exogenous as well as endogenous IL-6 and IL-1 regulate neuronal excitability, long-term potentiation and learning (Jankowsky and Patterson, 1999; Balschun et al., 2004; Bauer et al., 2007). IL-6 and related cytokines also regulate the stress response, feeding, sleep and depressive behaviors in the adult brain, and injections of certain cytokines can induce psychiatric symptoms in adult humans (Capuron and Dantzer, 2003; Theoharides et al., 2004; Schiepers et al., 2005; Bauer et al., 2007). These considerations raise the possibility that treating the inflammation and lowering the level of inflammatory cytokines in the adult or young brain might be able to improve symptoms. In fact, a recent pilot study of 25 autistic children suggested that behavioral symptoms improve after treatment with

Pioglitazone, an anti-inflammatory drug that is especially active against microglia (Boris et al., 2007). It should be noted, however, that this trial was not placebo-controlled and outcome was based on parental reporting. A clinical trail of the anti-inflammatory drug minocycline is ongoing at the NIH.

MIA has the potential to cause an ongoing inflammatory process such as is seen in autism. The developing immune system may need to find an appropriate balance between pro-and anti-inflammatory signaling, and MIA may permanently alter this setpoint. Such an alteration in development is seen in the offspring of pregnant rats that have been exposed to restraint stress throughout pregnancy. Offspring of stressed females have an altered behavioral response to stress as adults, which correlates with a significantly longer time for serum corticosterone to return to baseline following HPA axis activation. This alteration is due to early exposure to elevated glucocorticoid, as adrenalectomy of the mothers prevents the changes in the offspring, and administration of a synthetic glucocorticoid, dexamethasone, induces the changes (Barbazanges et al., 1996). Importantly, maternal stress alters corticosteroid receptor expression in the hippocampus of the adult offspring, an area important for terminating the stress response (Barbazanges et al., 1996; Levitt et al., 1996; Francis et al., 1999). A mechanism whereby early exposure to a biological signal causes an alteration in the later response to that signal could be applicable to the immune system.

In fact, an early postnatal inflammatory challenge can cause the organism to respond differently to subsequent challenges in adulthood. Rats exposed to LPS on P14 show a blunted febrile response to LPS as adults, and abnormally high COX-2 levels under baseline conditions, which are reduced after LPS challenge (normal animals show

the opposite: low COX-2 levels under baseline conditions, which increase upon LPS stimulation)(Boisse et al., 2004). In a similar study, rats given a neonatal *E. coli* infection show impaired memory and increased brain inflammation after a subsequent challenge with LPS as adults (Bilbo et al., 2005a; Bilbo et al., 2005b), compared to control rats given only an adult LPS injection. Maternal immune challenge may have a similar programming effect on the fetus as these early post-natal examples. The offspring of LPS-treated mothers display blunted or absent responses to a pre-weaning LPS injection (Hodyl et al., 2007; Lasala and Zhou, 2007), and fewer circulating monocytes are present in the adult offspring (Hodyl et al., 2007). MIA can also affect the way that the brain responds to subsequent non-immunologic challenges. Wang et al. (2007) injected LPS into the uterus of E15 mice, and then induced hypoxia-ischemia in neonatal or adult offspring. They report that in neonatal (P5 or 9) mice, ischemia was more severe in LPS-exposed mice than in controls, whereas in adult ischemia, LPS exposure was protective (Wang et al., 2007).

Perhaps alteration of the fetal immune system, such that it is hyper-responsive to later challenges, could account for the frequent anecdotal connections between regressive autism and illness at the time of regression. The sudden onset of autistic symptoms in children and adults has been reported following encephalitis or infection with herpes simplex, varicella, cytomegalovirus (Libbey et al., 2005) and malaria (Mankoski et al., 2006). CNS infections of this type are known to rapidly induce pro-inflammatory cytokine expression. In contrast, infections in autistic children are associated with acute amelioration of of behavioral symptoms, consistent with ongoing regulation of behavior by cytokines (Curran et al., 2007).

As with the behavioral data, cytokines probably play a large role in mediating the long-term immunologic effects of MIA. In a recent study involving the response of mice to maternal IL-2, daily injections of IL-2 from E12-17 resulted in elevated B and T cell counts in response to antigenic stimulation in pre-weaning pups. The results were interpreted as an acceleration of T cell development and a skewing of TH responses towards TH-1 (Ponzio et al., 2007). Moreover, a recent preliminary report showed that IL-1 administration to neonatal rats triggers microglial activation in the brain that persists into adulthood, and which is accompanied by a PPI deficit. Remarkably, treatment with the anti-inflammatory drug minocycline normalized the levels of microglia in the brain as well as the PPI (Tsuda et al., 2007).

It would be informative to develop an MIA model in mice with inflammation similar to that observed in autism, namely increased inflammatory cytokines in CSF, and microglial and astrocyte activation (Vargas et al., 2005). To date, most groups have reported only mild increases or no changes in inflammatory parameters in the adult rodent brain. The exception is a recent report (Romero et al., 2007) using a severe protocol of daily LPS administration throughout rat pregnancy. This protocol yields strikingly elevated serum levels of IL-1β, IL-2, IL-6 and TNF-α in the adult offspring. The high serum levels of cytokines would be expected to have dramatic consequences on the behavior of these animals. The only behavior that the authors assayed, PPI, is abnormal in the exposed offspring. Remarkably, both serum levels of inflammatory cytokines, as well as PPI are normalized by treatment of the adult offspring with the antipsychotic drug haloperidol. In addition, even two weeks after ending haloperidol treatment, the levels of IL-6, IL-2 and IL-12 remain lower than untreated, prenatally-

exposed animals. This study demonstrates not only that MIA can alter both behavior and immunological parameters in the adult offspring, but also that behavior and immunological parameters are tightly correlated. Coupled with the neonatal IL-1 data showing behavioral improvements in adult animals after anti-inflammatory treatment (Tsuda et al., 2007), these studies highlight the potential for treatment of abnormal behavior through normalization of inflammatory cytokines.

Finally, it is also of interest that Piontkewitz et al. (2007) have recently reported that treatment of adult offspring of poly(I:C)-treated pregnant rats with the anti-psychotic drug clozapine normalizes both behavioral abnormalities and ventricular dilation as observed by structural magnetic resonance imaging. Moreover, these positive effects can also be achieved during the prodromal period, before the post-pubertal onset of pathology and behavior. This result suggests the possibility of preventative treatment, highlighting the potential clinical relevance of the MIA model.

Conclusions and Perspectives

Schizophrenia and autism are thought to result from an interaction of a susceptibility genotype and environmental risk factors. The recent trend to focus on susceptibility genes has yielded some interesting candidates, and study of environmental factors that interact with those genes could reveal much about pathogenesis, prevention and potential treatments. Maternal infection is an environmental risk factor for both schizophrenia and autism, and a preponderance of evidence suggests that the maternal immune system, rather than pathogen invasion of the fetus, is detrimental. Several animal models of MIA are now well characterized, and these models mimic diverse

behavioral and pathological symptoms of the disorders. Coupled with their etiological relevance, these models offer attractive research opportunities.

Although full description of the MIA models continues, work has recently begun on the second phase of study: dissecting the mechanisms of how MIA alters fetal brain development, and the long-term changes in immune status that are set in motion by MIA. Regarding the former issue, maternal IL-6 produced in response to infection likely crosses the placenta and interacts with the fetal brain, although it may also alter the placenta itself. Ongoing research is examining the site of IL-6 action, with the eventual goal of characterizing the cellular and molecular changes caused by MIA. Regarding the permanent immune dysregulation in the postnatal brain, recent reports have shown that antipsychotic drugs can suppress an over-active immune system caused by MIA, and treatment with anti-inflammatory (or anti-psychotic drugs) can normalize behavioral abnormalities produced by neonatal or MIA-induced inflammatory cytokine exposure. A more thorough understanding of the mechanisms relating MIA with later psychiatric disease will hopefully lead to better prevention and treatment of these devastating disorders.

References	Species and Treatment	Behavioral Findings	Histological Findings
(Cai et al., 2000)	4 mg/kg LPS I.P. E18,19 rat P8 histology	N.R.	Increased GFAP, decreased MBP, altered microglial staining
(Ling et al., 2004)	10,000EU/kg LPS I.P. E10.5 rat Adult (>1yr) histology	N.R.	Fewer TH+ neurons and increased microglial staining in substantia nigra
(Borrell et al., 2002)	1 mg/kg LPS S.C. Alternate days throughout rat pregnancy; Adult histology	PPI deficits corrected by antipsychotic drugs	Increased GFAP, MHCII staining of microglia, TH increase in nucl accumb
(Bakos et al., 2004)	20-80 ug/kg LPS S.C E15-19 rat (increasing dose schedule)	Increased entries into all arms of plus maze, slips in beam walking test.	N.R.
(Fortier et al., 2004)	50ug/kg LPS I.P.	Increased amph.-induced locomotion, acoustic startle	N.R.

2004)	E18 & 19 rat	locomotion, acoustic startle response	
(Paintlia et al., 2004)	1 mg/kg LPS I.P E18 rat E20 or P9-30 Histology	N.R.	Less MBP, PLP and myelin staining at P9-30, more microglia at E20
(Golan et al., 2005; Golan et al., 2006)	0.12 mg/kg LPS I.P. E17 mouse Adult histology	Normal exploration and motor function, mostly normal learning/memory but specific deficits	Smaller, denser neurons in hippocampus, more pyknotic cells in cortex
(Fatemi et al., 2002; Shi et al., 2003; Shi et al., Submitted)	Intranasal influenza E9 mouse Adult histology	PPI, open field, novel object, social interaction deficits	Large adult brain, pyramidal cell atrophy, Purkinje cell deficit
(Smith et al., 2007; Shi et al., Submitted)	20mg/kg poly(I:C) I.P. E12 mouse Adult histology	PPI, LI,open field, social interaction deficits	Purkinje cell deficit
(Zuckerman et al., 2003; Zuckerman and	4mg/kg poly(I:C) I.V. E15 rat	LI deficit, enhanced reversal learning, normal water maze, increased amph. and MK-	Pyknotic cells in hippocampus, increased KCl-stimulated dopamine release

Zuckerman and Weiner, 2005)	Adult histology	increased amph. and MK-801 locomotion	stimulated dopamine release in striatum
(Meyer et al., 2006a; Nyffeler et al., 2006)	5 mg/kg poly(I:C) I.V. E9 mouse Adult histology	PPI, LI, open field, working memory deficits; increased amph-induced locomotion	GABAA receptor increase, no increase in pyknotic cells in hippocampus
(Ozawa et al., 2006)	5 mg/kg poly(I:C) I.P. Daily E12-17 mouse Adult histology	PPI, open field, working memory deficit, increased amph locomotion	Altered dopamine metabolism in striatum

Table I: Behavior and histology outcomes following various types of MIA. Different rows represent different research groups. Amph, amphetamine; GABA, g-aminobutyric acid; GFAP, glial fibrillary acidic protein; I.P, intraperitoneal; I.V., intravenous; MBP, myelin basic protein; N.R., not reported; PLP, proteolipid protein; PPI, prepulse inhibition; LI, latent inhibition; LPS, lipopolysaccharide; nucl accumb, shell of nucleus accumbens; S.C., subcutaneous; TH, tyrosine hydroxylase

Reference	Treatment	Findings
(Cai et al., 2000)*	4 mg/kg LPS I.P. E18 rat	TNFα, IL1β increased
(Urakubo et al., 2001)	2.5 mg/kg LPS I.P. E16 rat	TNFα increased
(Paintlia et al., 2004)*	1 mg/kg LPS I.P.	TNFα, IL1β, iNOS increased
(Ashdown et al., 2006)	0.05 mg/kg LPS I.P. E18 rat	No change in TNFα, IL1β, IL6
(Gilmore et al., 2005)	20 mg/kg poly(I:C) I.P. E16 rat	No change in TNFα
(Golan et al., 2005)	0.12 mg/kg LPS I.P. E17 mouse	IL6 increased
(Meyer et al., 2006a)	5 mg/kg poly(I:C) I.V. E9 mouse	IL1β, IL6 increased
(Meyer et al., 2006b)	5 mg/kg poly(I:C) I.V. E17 mouse	IL1β, IL6, IL10 increased

(Liverman et al., 2006)*	50ug LPS I.P. E18 mouse	IL1β, IL6, MCP-1, VEGF increased
(Meyer et al., 2007)	2 mg/kg poly(I:C) I.V. E9 mouse	TNFα, IL1β, IL6, IL10 increased

Table II. **Maternal immune activation increases cytokine levels in the fetal brain**. Assays were for cytokine protein, except where noted (*mRNA assayed). 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)) which may not produce detectable changes.

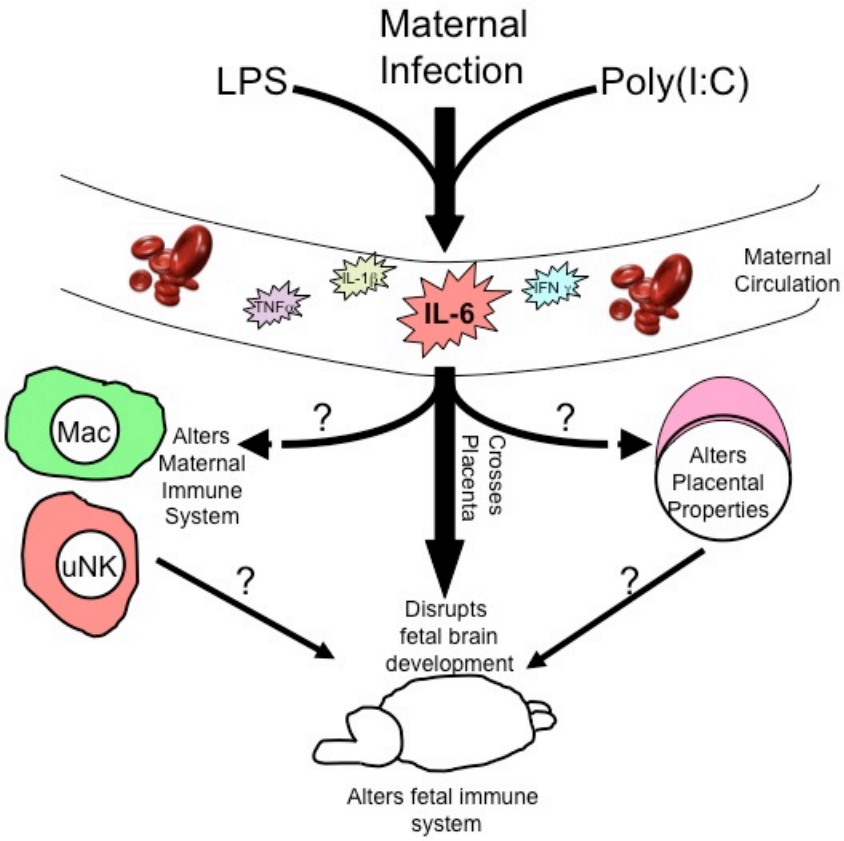

Figure 1: Proposed mechanism through which MIA leads to behavior abnormalities. Maternal infection, LPS, or poly(I:C) all lead to increased levels of cytokines in the maternal circulation. The cytokine IL-6 disrupts fetal brain development, either by crossing the placenta and directly interfering with signaling pathways in the developing brain, or indirectly via alterations to the placenta or the maternal immune system.

References

Akshoomoff N, Lord C, Lincoln AJ, Courchesne RY, Carper RA, Townsend J, Courchesne E (2004) Outcome classification of preschool children with autism spectrum disorders using MRI brain measures. J Am Acad Child Adolesc Psychiatry 43:349-357.

Arion D, Unger T, Lewis DA, Levitt P, Mirnics K (2007) Molecular evidence for increased expression of genes related to immune and chaperone function in the prefrontal cortex in schizophrenia. Biol Psychiatry 62:711-721.

Ashdown H, Dumont Y, Ng M, Poole S, Boksa P, Luheshi GN (2006) The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia. Mol Psychiatry 11:47-55.

Babulas V, Factor-Litvak P, Goetz R, Schaefer CA, Brown AS (2006) Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. Am J Psychiatry 163:927-929.

Bagalkote H, Pang D, Jones P (2001) Maternal influenza and schizophrenia in the offspring. Intl J Ment Health 39:3-21.

Bakos J, Duncko R, Makatsori A, Pirnik Z, Kiss A, Jezova D (2004) Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. Ann N Y Acad Sci 1018:281-287.

Balschun D, Wetzel W, Del Rey A, Pitossi F, Schneider H, Zuschratter W, Besedovsky HO (2004) Interleukin-6: a cytokine to forget. Faseb J 18:1788-1790.

Barbazanges A, Piazza PV, Le Moal M, Maccari S (1996) Maternal glucocorticoid secretion mediates long-term effects of prenatal stress. J Neurosci 16:3943-3949.

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. Nat Rev Neurosci 8:221-232.

Bell MJ, Hallenbeck JM, Gallo V (2004) Determining the fetal inflammatory response in an experimental model of intrauterine inflammation in rats. Pediatr Res 56:541-546.

Beloosesky R, Gayle DA, Amidi F, Nunez SE, Babu J, Desai M, Ross MG (2006) N-acetyl-cysteine suppresses amniotic fluid and placenta inflammatory cytokine responses to lipopolysaccharide in rats. Am J Obstet Gynecol 194:268-273.

Bilbo SD, Biedenkapp JC, Der-Avakian A, Watkins LR, Rudy JW, Maier SF (2005a) Neonatal infection-induced memory impairment after lipopolysaccharide in adulthood is prevented via caspase-1 inhibition. J Neurosci 25:8000-8009.

Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF (2005b) Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neurosci 119:293-301.

Bobetsis YA, Barros SP, Offenbacher S (2006) Exploring the relationship between periodontal disease and pregnancy complications. Journal of the American Dental Association 137:7s-13s.

Boisse L, Mouihate A, Ellis S, Pittman QJ (2004) Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci 24:4928-4934.

Boris M, Kaiser CC, Goldblatt A, Elice MW, Edelson SM, Adams JB, Feinstein DL (2007) Effect of pioglitazone treatment on behavioral symptoms in autistic children. J Neuroinflammation 4:3.

Borrell J, Vela JM, Arevalo-Martin A, Molina-Holgado E, Guaza C (2002) Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. Neuropsychopharmacology 26:204-215.

Brown AS, Schaefer CA, Quesenberry CP, Jr., Liu L, Babulas VP, Susser ES (2005) Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. Am J Psychiatry 162:767-773.

Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP, Susser ES (2004a) Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 61:774-780.

Brown AS, Hooton J, Schaefer CA, Zhang H, Petkova E, Babulas V, Perrin M, Gorman JM, Susser ES (2004b) Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry 161:889-895.

Cai Z, Pan ZL, Pang Y, Evans OB, Rhodes PG (2000) Cytokine induction in fetal rat brains and brain injury in neonatal rats after maternal lipopolysaccharide administration. Pediatr Res 47:64-72.

Capuron L, Dantzer R (2003) Cytokines and depression: the need for a new paradigm. Brain Behav Immun 17 Suppl 1:S119-124.

Chess S (1977) Follow-up report on autism in congenital rubella. J Autism Child Schizophr 7:69-81.

Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M (2007) Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. Pediatr Neurol 36:361-365.

Ciaranello AL, Ciaranello RD (1995) The neurobiology of infantile autism. Annu Rev Neurosci 18:101-128.

Clancy B, Darlington RB, Finlay BL (2001) Translating developmental time across mammalian species. Neuroscience 105:7-17.

Crawley JN (2007) Mouse behavioral assays relevant to the symptoms of autism. Brain Pathol 17:448-459.

Croonenberghs J, Bosmans E, Deboutte D, Kenis G, Maes M (2002) Activation of the inflammatory response system in autism. Neuropsychobiology 45:1-6.

Curran LK, Newschaffer CJ, Lee LC, Crawford SO, Johnston MV, Zimmerman AW (2007) Behaviors associated with fever in children with autism spectrum disorders. Pediatrics 120:e1386-1392.

Dahlgren J, Samuelsson AM, Jansson T, Holmang A (2006) Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr Res 60:147-151.

Dammann O, Kuban KC, Leviton A (2002) Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born preterm. Ment Retard Dev Disabil Res Rev 8:46-50.

Davis JO, Phelps JA, Bracha HS (1995) Prenatal development of monozygotic twins and concordance for schizophrenia. Schizophr Bull 21:357-366.

Desmond MM, Wilson GS, Melnick JL, Singer DB, Zion TE, Rudolph AJ, Pineda RG, Ziai MH, Blattner RJ (1967) Congenital rubella encephalitis. Course and early sequelae. J Pediatr 71:311-331.

Elovitz MA, Mrinalini C, Sammel MD (2006) Elucidating the early signal transduction pathways leading to fetal brain injury in preterm birth. Pediatr Res 59:50-55.

Fatemi SH, Sidwell R, Kist D, Akhter P, Meltzer HY, Bailey K, Thuras P, Sedgwick J (1998) Differential expression of synaptosome-associated protein 25 kDa [SNAP-25] in hippocampi of neonatal mice following exposure to human influenza virus in utero. Brain Res 800:1-9.

Fatemi SH, Earle J, Kanodia R, Kist D, Emamian ES, Patterson PH, Shi L, Sidwell R (2002) Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia. Cell Mol Neurobiol 22:25-33.

Fatemi SH, Emamian ES, Kist D, Sidwell RW, Nakajima K, Akhter P, Shier A, Sheikh S, Bailey K (1999) Defective corticogenesis and reduction in Reelin

immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. Mol Psychiatry 4:145-154.

Fortier ME, Joober R, Luheshi GN, Boksa P (2004) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res 38:335-345.

Francis D, Diorio J, Liu D, Meaney MJ (1999) Nongenomic transmission across generations of maternal behavior and stress responses in the rat. Science 286:1155-1158.

Garbett K, Ebert P, Lintas C, Mirnics K, Persico A (2007) Immune transcript disturbances in temporal cortex of autistic brains. 2007 Society for Neuroscience poster presentation.

Garver DL, Tamas RL, Holcomb JA (2003) Elevated interleukin-6 in the cerebrospinal fluid of a previously delineated schizophrenia subtype. Neuropsychopharmacology 28:1515-1520.

Gilmore JH, Jarskog LF, Vadlamudi S (2005) Maternal poly I:C exposure during pregnancy regulates TNF alpha, BDNF, and NGF expression in neonatal brain and the maternal-fetal unit of the rat. J Neuroimmunol 159:106-112.

Gilmore JH, Jarskog LF, Vadlamudi S, Lauder J (2004) Prenatal infection and risk for schizophrenia: IL-I beta, IL-6, and TNF alpha inhibit cortical neuron dendrite development. Neuropsychopharmacology 29:1221-1229.

Golan H, Stilman M, Lev V, Huleihel M (2006) Normal aging of offspring mice of mothers with induced inflammation during pregnancy. Neuroscience 141:1909-1918.

Golan HM, Lev V, Hallak M, Sorokin Y, Huleihel M (2005) Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy. Neuropharmacology 48:903-917.

Han YW, Redline RW, Li M, Yin L, Hill GB, McCormick TS (2004) Fusobacterium nucleatum induces premature and term stillbirths in pregnant mice: implication of oral bacteria in preterm birth. Infect Immun 72:2272-2279.

Hava G, Vered L, Yael M, Mordechai H, Mahoud H (2006) Alterations in behavior in adult offspring mice following maternal inflammation during pregnancy. Dev Psychobiol 48:162-168.

Hodyl NA, Krivanek KM, Lawrence E, Clifton VL, Hodgson DM (2007) Prenatal exposure to a pro-inflammatory stimulus causes delays in the development of the innate immune response to LPS in the offspring. J Neuroimmunol 190:61-71.

Hyman SL, Arndt TL, Rodier PM (2005) Environmental Agents and Autism: Once and Future Associations. International Review of Research in Mental Retardation Volume 30:171-194.

Jankowsky JL, Patterson PH (1999) Cytokine and growth factor involvement in long-term potentiation. Mol Cell Neurosci 14:273-286.

Lasala N, Zhou H (2007) Effects of maternal exposure to LPS on the inflammatory response in the offspring. J Neuroimmunol 189:95-101.

Lemery-Chalfant K, Goldsmith HH, Schmidt NL, Arneson CL, Van Hulle CA (2006) Wisconsin Twin Panel: current directions and findings. Twin Res Hum Genet 9:1030-1037.

Levitt NS, Lindsay RS, Holmes MC, Seckl JR (1996) Dexamethasone in the last week of pregnancy attenuates hippocampal glucocorticoid receptor gene expression and elevates blood pressure in the adult offspring in the rat. Neuroendocrinology 64:412-418.

Libbey JE, Sweeten TL, McMahon WM, Fujinami RS (2005) Autistic disorder and viral infections. J Neurovirol 11:1-10.

Lin D, Smith MA, Champagne C, Elter J, Beck J, Offenbacher S (2003a) Porphyromonas gingivalis infection during pregnancy increases maternal tumor necrosis factor alpha, suppresses maternal interleukin-10, and enhances fetal growth restriction and resorption in mice. Infect Immun 71:5156-5162.

Lin D, Smith MA, Elter J, Champagne C, Downey CL, Beck J, Offenbacher S (2003b) Porphyromonas gingivalis infection in pregnant mice is associated with placental dissemination, an increase in the placental Th1/Th2 cytokine ratio, and fetal growth restriction. Infect Immun 71:5163-5168.

Ling Z, Chang QA, Tong CW, Leurgans SE, Lipton JW, Carvey PM (2004) Rotenone potentiates dopamine neuron loss in animals exposed to lipopolysaccharide prenatally. Exp Neurol 190:373-383.

Liverman CS, Kaftan HA, Cui L, Hersperger SG, Taboada E, Klein RM, Berman NE (2006) Altered expression of pro-inflammatory and developmental genes in the fetal brain in a mouse model of maternal infection. Neurosci Lett 399:220-225.

Mankoski RE, Collins M, Ndosi NK, Mgalla EH, Sarwatt VV, Folstein SE (2006) Etiologies of autism in a case-series from Tanzania. J Autism Dev Disord 36:1039-1051.

Mednick SA, Machon RA, Huttunen MO, Bonett D (1988) Adult schizophrenia following prenatal exposure to an influenza epidemic. Arch Gen Psychiatry 45:189-192.

Meyer U, Feldon J, Schedlowski M, Yee BK (2006a) Immunological stress at the maternal-foetal interface: a link between neurodevelopment and adult psychopathology. Brain Behav Immun 20:378-388.

Meyer U, Murray PJ, Urwyler A, Yee BK, Schedlowski M, Feldon J (2007) Adult behavioral and pharmacological dysfunctions following disruption of the fetal brain balance between pro-inflammatory and IL-10-mediated anti-inflammatory signaling. Mol Psychiatry.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006b) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Morgan JT, Chana G, Buckwalter J, Courchesne E, Everall IP (2007) Increased Iba-1 positive microglial cell denisty in the autistic brain. 2007 Society for Neuroscience poster presentation.

Nawa H, Takei N (2006) Recent progress in animal modeling of immune inflammatory processes in schizophrenia: implication of specific cytokines. Neurosci Res 56:2-13.

Nyffeler M, Meyer U, Yee BK, Feldon J, Knuesel I (2006) Maternal immune activation during pregnancy increases limbic GABAA receptor immunoreactivity in the adult offspring: implications for schizophrenia. Neuroscience 143:51-62.

O'Callaghan E, Sham PC, Takei N, Murray G, Glover G, Hare EH, Murray RM (1994) The relationship of schizophrenic births to 16 infectious diseases. Br J Psychiatry 165:353-356.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Paintlia MK, Paintlia AS, Barbosa E, Singh I, Singh AK (2004) N-acetylcysteine prevents endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in developing rat brain. J Neurosci Res 78:347-361.

Palmen SJ, van Engeland H, Hof PR, Schmitz C (2004) Neuropathological findings in autism. Brain 127:2572-2583.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Patterson PH (2005) Maternal influenza infection leads to neuropathology and behavioral abnormalities in adult offspring. Neuropsychopharmacology 30:S9-S9.

Paul R, Koedel U, Winkler F, Kieseier BC, Fontana A, Kopf M, Hartung HP, Pfister HW (2003) Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis. Brain 126:1873-1882.

Perry W, Minassian A, Lopez B, Maron L, Lincoln A (2006) Sensorimotor Gating Deficits in Adults with Autism. Biol Psychiatry.

Phelps JA, Davis JO, Schartz KM (1997) Nature, Nurture, and Twin Research Strategies. Current Directions in Psychological Science 6:117-121.

Pierce K, Courchesne E (2001) Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. Biol Psychiatry 49:655-664.

Piontkewitz Y, Weiner I, Assaf Y (2007) Post-pubertal emergence of schizophrenia-like abnormalaties following prenatal immune system activation and thier prevention:

Modeling the disorder and its prodrome. In: 7th IBRO World Congress of Neuroscience, p 45. Melbourne, Australia.

Ponzio NM, Servatius R, Beck K, Marzouk A, Kreider T (2007) Cytokine levels during pregnancy influence immunological profiles and neurobehavioral patterns of the offspring. Ann N Y Acad Sci 1107:118-128.

Relier JP (2001) Influence of maternal stress on fetal behavior and brain development. Biol Neonate 79:168-171.

Robertson SA, Skinner RJ, Care AS (2006) Essential role for IL-10 in resistance to lipopolysaccharide-induced preterm labor in mice. J Immunol 177:4888-4896.

Romero E, Ali C, Molina-Holgado E, Castellano B, Guaza C, Borrell J (2007) Neurobehavioral and immunological consequences of prenatal immune activation in rats. Influence of antipsychotics. Neuropsychopharmacology 32:1791-1804.

Saetre P, Emilsson L, Axelsson E, Kreuger J, Lindholm E, Jazin E (2007) Inflammation-related genes up-regulated in schizophrenia brains. BMC Psychiatry 7:46.

Saliba E, Henrot A (2001) Inflammatory mediators and neonatal brain damage. Biol Neonate 79:224-227.

Samuelsson AM, Jennische E, Hansson HA, Holmang A (2006) Prenatal exposure to interleukin-6 results in inflammatory neurodegeneration in hippocampus with NMDA/GABA(A) dysregulation and impaired spatial learning. Am J Physiol Regul Integr Comp Physiol 290:R1345-1356.

Sargent IL, Borzychowski AM, Redman CW (2006) NK cells and human pregnancy--an inflammatory view. Trends Immunol 27:399-404.

Schiepers OJ, Wichers MC, Maes M (2005) Cytokines and major depression. Prog Neuropsychopharmacol Biol Psychiatry 29:201-217.

Shi L, Tu N, Patterson PH (2005) Maternal influenza infection is likely to alter fetal brain development indirectly: the virus is not detected in the fetus. Int J Dev Neurosci 23:299-305.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 23:297-302.

Shi L, Smith SE, Malkova N, Tse D, Patterson PH (Submitted) Activation of the maternal immune system alters cerebellar development in the offspring.

Singh VK, Warren RP, Odell JD, Cole P (1991) Changes of soluble interleukin-2, interleukin-2 receptor, T8 antigen, and interleukin-1 in the serum of autistic children. Clin Immunol Immunopathol 61:448-455.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Theoharides TC, Weinkauf C, Conti P (2004) Brain cytokines and neuropsychiatric disorders. J Clin Psychopharmacol 24:577-581.

Tsuda N, Eda T, Mizuno M, Sotoyama H, Nawa H (2007) Minocycline improves cognitive and behavioral impairments resulted from neonatal exposure to interleukin-1. 2007 Society for Neuroscience poster presentation.

Turetsky BI, Calkins ME, Light GA, Olincy A, Radant AD, Swerdlow NR (2007) Neurophysiological Endophenotypes of Schizophrenia: The Viability of Selected Candidate Measures. Schizophr Bull 33:69-94.

Urakubo A, Jarskog LF, Lieberman JA, Gilmore JH (2001) Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophr Res 47:27-36.

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57:67-81.

Wang X, Rousset CI, Hagberg H, Mallard C (2006) Lipopolysaccharide-induced inflammation and perinatal brain injury. Semin Fetal Neonatal Med 11:343-353.

Wang X, Hagberg H, Nie C, Zhu C, Ikeda T, Mallard C (2007) Dual role of intrauterine immune challenge on neonatal and adult brain vulnerability to hypoxia-ischemia. J Neuropathol Exp Neurol 66:552-561.

Watson CG, Kucala T, Tilleskjor C, Jacobs L (1984) Schizophrenic birth seasonality in relation to the incidence of infectious diseases and temperature extremes. Arch Gen Psychiatry 41:85-90.

Weiner I (2003) The "two-headed" latent inhibition model of schizophrenia: modeling positive and negative symptoms and their treatment. Psychopharmacology (Berl) 169:257-297.

Weinstock M (2001) Alterations induced by gestational stress in brain morphology and behaviour of the offspring. Prog Neurobiol 65:427-451.

Zhang XY, Zhou DF, Cao LY, Wu GY, Shen YC (2005) Cortisol and cytokines in chronic and treatment-resistant patients with schizophrenia: association with psychopathology and response to antipsychotics. Neuropsychopharmacology 30:1532-1538.

Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A, Heyes MP (2005) Cerebrospinal fluid and serum markers of inflammation in autism. Pediatr Neurol 33:195-201.

Zuckerman L, Weiner I (2005) Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. J Psychiatr Res 39:311-323.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Chapter 3

Maternal Immune Activation Alters Fetal Brain Development Through Interleukin-6

Stephen E.P. Smith[1], Jennifer Li[1,2], Krassimira Garbett[3], Karoly Mirnics[3] and Paul H. Patterson[1*]

[1]Biology Division, California Institute of Technology, Pasadena, CA

[2]Present address: Dept. of Physiology, Univ. of Calif. Medical Center, San Francisco, CA

[3] Dept. of Psychiatry and Vanderbilt Kennedy Center for Human Development, Vanderbilt University, Nashville, TN

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Abstract

Schizophrenia and autism are thought to result from the interaction between a susceptibility genotype and environmental risk factors. The offspring of women who experience infection while pregnant have an increased risk for these disorders. Maternal immune activation (MIA) in pregnant rodents produces offspring with abnormalities in behavior, histology and gene-expression that are reminiscent of schizophrenia and autism, making MIA a useful model of the disorders. However, the mechanism by which MIA causes long-term behavioral deficits in the offspring is unknown. Here we show that the cytokine interleukin-6 (IL-6) is critical for mediating the behavioral and transcriptional changes in the offspring. A single maternal injection of IL-6 on day 12.5 of mouse pregnancy causes pre-pulse inhibition (PPI) and latent inhibition (LI) deficits in the adult offspring. Moreover, co-administration of an anti-IL-6 antibody in the poly(I:C) model of MIA prevents the PPI, LI, exploratory and social deficits caused by poly(I:C), and normalizes the associated changes in gene expression in the brains of adult offspring. Finally, MIA in IL-6 knockout mice does not result in several of the behavioral changes seen in the offspring of wild type mice following MIA. The identification of IL-6 as a key intermediary should aid in the molecular dissection of the pathways whereby MIA alters fetal brain development, which can shed new light on the pathophysiological mechanisms that predispose to schizophrenia and autism.

Introduction

Birth in winter or spring months is an accepted risk factor for schizophrenia, and the preponderance of evidence suggests that the prevalence of influenza in winter months is responsible (Tochigi et al., 2004). Over 25 studies have analyzed schizophrenia incidence following influenza epidemics, and the majority have found an increased incidence among exposed offspring. More recently, Brown and colleagues (Brown and Susser, 2002; Brown et al., 2004; Brown, 2006) examined the medical records of over 12,000 pregnant women and found that second trimester respiratory infection increases the risk for schizophrenia in the offspring 3-7-fold. Because of the high prevalence of influenza infection, they estimate that 14-21% of schizophrenia cases are caused by maternal infection. These findings are further supported by an association between elevated cytokines or anti-influenza antibodies in maternal serum and schizophrenia in the offspring (Brown et al., 2004). Maternal infection may also play a role in the pathogenesis of autism (Patterson, 2002). These links are even more remarkable considering that the epidemiological studies are unable to screen for susceptibility genotype. Because of the strong genetic component in autism and schizophrenia, it is likely that only genetically susceptible individuals who were exposed to maternal infection would develop the disorder, suggesting that the risk associated with maternal infection may be considerably greater than 3-7-fold in susceptible individuals.

Several lines of evidence indicate that the maternal immune response, rather than direct infection of the fetus, is responsible for the increased incidence of schizophrenia and autism in the offspring of mothers who suffer infections during pregnancy (Patterson, 2005). First, human influenza infection is usually confined to the respiratory tract.

Moreover, in a mouse model, influenza infection during pregnancy produces behavioral deficits in the adult offspring (Shi et al., 2003) but we detected no virus in the fetuses (Shi et al., 2005). Most importantly, injecting pregnant rodents with either the double-stranded RNA, poly(I:C), or with bacterial lipopolysaccharide (LPS), which both induce strong innate immune responses in the absence of infection, produces behavioral and histological abnormalities in the adult offspring similar to those seen in the offspring of infected mothers (Shi et al., 2003; Zuckerman et al., 2003; Fortier et al., 2004a; Zuckerman and Weiner, 2005; Meyer et al., 2006; Ozawa et al., 2006). Following maternal immune activation (MIA) by influenza infection, LPS or poly(I:C), cytokine levels are altered in the maternal serum as well as the amniotic fluid, placenta and fetal brain (Fidel et al., 1994; Cai et al., 2000; Urakubo et al., 2001; Gayle et al., 2004; Paintlia et al., 2004; Gilmore et al., 2005; Ashdown et al., 2006; Beloosesky et al., 2006; Meyer et al., 2006; Xu et al., 2006). Since cytokines drive the innate immune response, they are logical candidates for disruption of fetal brain development.

We have examined several pro-inflammatory cytokines as potential mediators of the effects of MIA on fetal brain development. Using the behavior of adult offspring as the readout, we first tested whether injection of single cytokines in the uninfected mother can mimic the effects of maternal infection on the offspring. To then test the involvement of endogenous cytokines, we asked if anti-cytokine antibodies can block the effects of maternal poly(I:C) injection on the offspring, and whether MIA affects the behavior of offspring of cytokine knockout (KO) mice. The data identify IL-6 as a key mediator of the effects of MIA on fetal brain development.

Materials and Methods

Generation of animals. Female C57BL/6J mice (Jackson Laboratories, 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 E0. Pregnant females were not disturbed, except for weekly cage cleaning, until E12.5 when they were weighed and pseudo-randomly assigned to one of 7 groups. Each group initially contained at least 5 pregnant females.

Cytokine injection. Mice were injected i.p. with either 5 μg carrier protein-free recombinant mouse interleukin-6 (IL-6), or 5 μg carrier-free recombinant mouse interferon γ (IFNg) (R&D Systems, Minneapolis) freshly dissolved in 200 μl 0.9% saline. Control mice were injected with 200 μl vehicle. Injections in the mg range in adult mice cause acute behavioral effects (Swiergiel et al, 1997); our pilot experiments suggested that maternal doses in this range have a significant effect on adult offspring.

Cytokine blocking. Four groups of mice were injected i.p. with 20 mg/kg poly(I:C)(potassium salt, Sigma, St. Louis) freshly dissolved in saline, as previous experiments found this dose to be most effective with i.p. delivery (Shi et al, 2003). The manufacturer supplies poly(I:C) at 10% of the total weight of the salt, and dosage was based upon the weight of poly(I:C) itself. Some of the mice were co-injected with 100 μg cytokine-neutralizing antibody targeted against either interleukin-6 (anti-IL-6)(rat IgG_1), interferon-γ (anti-IFNγ)(rat IgG_{2a}) or interleukin-1β (anti-IL1β)(rat IgG_1)(R&D Systems), freshly dissolved in sterile saline. Total injection volume was 200 μl. The half-life of rat IgG_1 and $IgG2_a$ in pregnant mice is similar to that of mouse IgG, with an

α-phase lasting approximately 24 hours, and a β-phase lasting over 200 hours, with 2-3% maternofetal transmission (Medesan et al., 1998). The antibody dose was calculated to be approximately a 10-fold molar excess of the maximal cytokine levels observed in response to poly(I:C). Control mice were injected with either 200 μl saline or 100 mg of anti-IL6 freshly dissolved in 200 ml saline.

IL-6 KO mice. IL-6 KO mice, strain B6.129S2-IL6tm1Kopf/J, back-crossed for 11 generations onto a C57 background, were obtained from Jackson Labs and maintained in our facility by homozygous breeding. Mice were mated and injected with 20 mg/kg poly(I:C) or saline, using the procedures outlined above.

All pregnant mice were single-housed after injection, and returned to the same cage rack. Mice were left undisturbed except for weekly cage cleaning, until the pups were weaned at 3 weeks of age. Offspring were housed in same-sex groups of 2-5 animals.

Measurement of maternal cytokine levels. Pregnant females were injected with saline, poly(I:C), poly(I:C) + anti-IL-6 or poly(I:C) + anti-IFNγ, as above. Three hours after injection, mice were euthanized with an overdose of sodium pentobarbital (Nembutal) and blood was collected via cardiac puncture into Eppindorf tubes and allowed to clot at room temperature for 1 hr. Blood was centrifuged at 8000 x g for 10 min at 4^{o} C and the serum aliquoted and stored at –80 ^{o} until use. ELISAs for IL-6, IFN-γ and IL-1β (R&D Systems) were performed according to the manufacturers' instructions.

For immunoprecipitation of anti-cytokine antibodies, a biotinylated, anti-rat antibody that was pre-absorbed with mouse serum to prevent binding to mouse antibodies (Vector, Burlingame, CA) was conjugated to streptavidin magnetic beads (NEB, Ipswich,

MA) overnight at 4 °. After washing the beads thoroughly with PBS, mouse serum was diluted 1:20 in PBS and incubated with the beads for 4 hours at room temperature. The beads were removed magnetically, and the resulting serum was used directly for ELISA.

Cytokine detection array kits were purchased from RayBioTech (Norcross, GA) and manufacture's instructions were followed. Briefly, antibody-spotted membranes were treated with blocking and incubated overnight at 4° C with 50 µl of mouse serum to be tested, washed with wash buffer, probed with biotinylated anti-cytokine antibodies, and binding detected using strep-HRP chemiluminescence.

Behavioral Testing - Latent Inhibition. The protocol was modeled after Zuckerman and Weiner (2005). Each group of mice was randomly sub-divided into two groups, pre-exposed (PE) and not-pre-exposed (NPE). Mice were placed in a box (Coulborn Instruments, Allentown, PA) with a speaker mounted on the back wall and an infrared motion detector on the ceiling. PE mice were presented with 40 tones (2000 hz, 30 seconds (s) duration) separated by 30±40 s to randomize the inter-tone interval. NPE mice were placed in the same enclosure for an equivalent amount of time. Immediately following pre-exposure, all mice were given three pairing trials of the 30 s tone immediately followed by a 1 s, 0.3 mA foot shock delivered through the floor. Pairing trials were separated by 180 s. The next day, the mice were returned to the same enclosure for 8 min to measure context freezing (measured as described below). The following day, mice were again returned to the enclosure and, after a 180 s acclimation period, were presented with an 8 min tone presentation. Freezing during the tone presentation was measured by the sensors and defined as a period of ≥4 s during which movement was not detected. Data is presented as percent of the time spent freezing

during tone presentation, and latent inhibition (LI) is defined as the difference in the amount of freezing in response to the tone in PE mice compared to NPE mice.

Pilot experiments suggested that PE mice demonstrate a larger range of time spent freezing than NPE mice, so the groups were split unevenly (control-saline, 7 NPE mice and 20 PE mice; control-anti-IL-6, 7 and 15; poly(I:C), 6 and 13; poly(IC) + anti-IL-6, 10 and 29; poly(IC) + anti-IFNγ, 8 and 12; IL-6 7 and 10; IFNγ, 3 and 11), resulting in small numbers of NPE animals in some groups. Initially, the NPE animals belonging to different groups were treated as separate groups, but ANOVA revealed no significant differences between the groups [$F(6,41)=0.9241$, $p>>.05$] (Supplementary Fig. 1). Fear conditioning was therefore similar in all groups, and the NPE groups were merged for greater statistical power.

PPI. The prepulse inhibition (PPI) apparatus (San Diego Instruments, San Diego, CA) consists of a sound-insulated chamber with a speaker mounted on the ceiling. The subject is restrained in a plexiglass cylinder inside the chamber, and a pliezo-electric sensor is mounted beneath the restraining device to measure the startle response. After a 5 min acclimation period, the subject is presented with 6, 120 db pulses of white noise. The subject is then presented with 14 blocks of four different trial types in a pseudo-random order. Trial types include P5P, where a pre-pulse of 5 db above background (67 db), precedes the startle stimulus by 100 ms, P15P in which the prepulse is 15 db above background, startle stimulus alone, and no stimulus. Trials are averaged for each individual, and PPI is defined as PPI(X) = (Startle alone – PXP)/(Startle alone) where X = 5 or 15.

Open Field. Mice were placed in a 50 cm x 50 cm white plexiglass box brightly lit by florescent room lighting and 6, 60 w incandescent bulbs 4-6 feet above the box. Activity was recorded by a ceiling-mounted video camera and analyzed using Ethovision software (Noldus, Leesburg, VA). The software allows display of the paths taken by the mice, and it measures the total distance moved and the number of entries into the center of the arena (central 17 cm square) in a 10 min session.

Social Interaction. The testing apparatus consisted of a 60 cm x 40 cm plexiglass box divided into three chambers as described previously (Sankoorikal et al., 2006). Mice could move between chambers through a small opening (6 x 6 cm) in the dividers. Plastic cylinders in each of the two side chambers contained the probe mice, and numerous 1 cm holes in the cylinders enabled test and probe mice to contact each other. Mice to be tested were placed in the center chamber and an overhead camera recorded their movements. Mice were allowed 5 min to explore the box, after which an unfamiliar, same-sex probe mouse from the same experimental group was placed in one of two restraining cylinders. The Ethovision program measured time spent in each of the three chambers, and social preference was defined as (% time spent in the social chamber) – (% time spent in the opposite chamber).

DNA microarray analysis. Mice were killed by cervical dislocation and brains removed quickly. The olfactory bulb was peeled back and removed, and a 2 mm coronal section of cortex was removed from the front of the brain with a clean razor blade. This section was placed in a 1.5 ml tube and frozen in liquid nitrogen. Total RNA was isolated using the Trizol reagent (Invitrogen, Carlsbad, CA). RNA quality was assessed using the Agilent Bioanalyzer. Reverse transcription, *in vitro* transcription and fragmentation were

performed according to manufacturer's recommendations (Affymetrix, Sunnyvale, CA). Samples were hybridized onto MOE430A mouse Affymetrix GeneChips that contained >22,000 probesets using the Affymetrix hybridization station. To avoid microarray batch variation, only microarrays from a single lot were used. Microarrays were considered for use only if the average 3':5' ratio for GAPDH and actin did not exceed 1:1.2. Segmentation of scanned microarray images was performed by Microarray Analysis Suite 5.0 (MAS5). Determination of expression levels and scaling were performed using Robust Multi-array Average (RMA) (Irizarry et al., 2003a; Irizarry et al., 2003b). For scale linearity, the data were log2 transformed, and differential expression was established using average log2 ratio (ALR) between the studied cohorts (|ALR|=1 corresponds to a 2-fold increase or decrease, |ALR|= 0.585 represents a 50% change, while |ALR|= 0.263 depicts a 20% expression alteration).

Statistical Analysis. All data are shown as mean ± SEM. The statistical significance of differences between two groups was assessed using the Student's T-test, and differences among multiple groups was assessed using one-way ANOVAs followed by Bonferroni post-hoc tests.

Results

Effects of exogenous cytokines. Our pilot studies indicated that maternal administration of IL-6, but not IL-1a, TNFa or IFNg, causes prepulse inhibition (PPI) deficits in the adult offspring. PPI is the inhibition of a startle response when the startling stimulus is immediately preceded by a smaller, non-startling stimulus of the same modality, and is a measure of sensory-motor gating, attention and distractibility. PPI deficits are observed in several mental disorders, including schizophrenia (Wynn et al.,

2004) and autism (Perry et al., 2006). Furthermore, PPI deficits in the offspring elicited by maternal influenza infection respond to antipsychotic and psychomimetic drugs (Shi et al., 2003), and the PPI deficit resulting from poly(I:C) MIA is present in adult, but not juvenile rats (Zuckerman et al., 2003), mimicking the adult onset of schizophrenia. The changes seen in this very relevant behavior prompted further study of the effects of maternal IL-6 administration.

In a large follow-up study (see Supplementary Table 1 for numbers of animals), we confirmed that the offspring of pregnant mice injected with 5 mg of IL-6 on E12.5 display lower PPI than either control offspring ($p < 0.01$) or offspring of mothers injected with 5 mg of IFNg ($p < 0.05$) (Fig. 1a).

Both control offspring and offspring of mice injected with IFNg display significant latent inhibition (LI) compared to NPE mice, ($p < 0.001$ and $p < 0.05$, respectively). The offspring of IL-6-injected mice lack significant LI ($p > .05$ vs. NPE) (Fig. 1b). LI refers to the inhibition of a conditioned response to a stimulus when an individual has been repeatedly exposed to the stimulus before pairing with the unconditioned response. LI is a measure of the ability to ignore irrelevant stimuli, and its disruption is considered to be pertinent for the cognitive deficits in schizophrenia (Weiner, 2003). LI is disrupted in schizophrenic subjects and in amphetamine-treated humans and rats, restored to normal levels in schizophrenics by neuroleptic drugs, and enhanced in normal humans and rats by antipsychotic drugs (Weiner, 2003). As with PPI, MIA in rats disrupts LI in adult, but not pre-pubertal, offspring (Zuckerman et al., 2003) and the disrupted LI of poly(I:C)-exposed offspring responds to anti-psychotic drugs (Zuckerman and Weiner, 2005). Disruption of LI, enhanced amphetamine-induced locomotion (Zuckerman et al., 2003;

Ozawa et al., 2006) and altered concentrations of dopamine and its metabolites (Ozawa et al., 2006) caused by poly(I:C) MIA, are consistent with a subcortical dopamine dysfunction that is a central feature of many theories of schizophrenia (Weiner, 2003). Thus, a single injection of IL-6 on E12.5 causes deficits in two relevant behaviors (LI and PPI) in the adult offspring.

Effects of anti-IL-6 on abnormal behaviors evoked by MIA. To test the role of endogenous IL-6 during MIA, we co-administered an IL-6-neutralizing antibody with poly(I:C) on E12.5. To control for non-specific effects of antibody administration, we confirmed that a single maternal injection of anti-IL-6 antibody alone does not cause behavioral changes in the offspring (PPI, LI and open field; data not shown). Furthermore, maternal co-administration of poly(I:C) and neutralizing antibodies of the same, or different, Ig subclass (IL-1β/IgG_1 or anti-IFNγ/IgG_{2a}) produces offspring that behave similarly to the offspring of mice injected with poly(I:C) alone, indicating the lack of non-specific effects (see below). To ensure the efficacy of the antibodies, we measured cytokine levels in each treatment group by ELISA and cytokine array assay. We find that IL-6 and IL-1b are elevated in mice injected with poly(I:C), and that anti-IL-6 binds 98% of the total serum IL-6 induced by poly(I:C), without significantly altering the levels of other cytokines (Supplementary Fig. 2).

We tested two separate, large sets of offspring for several relevant behaviors as adults. Offspring of mice injected with poly(I:C) lack LI, as do the offspring of mice injected with poly(I:C) + anti-IFNγ neutralizing antibody (Fig. 2a). In contrast, mice injected with poly(I:C) + anti-IL-6 antibody show normal LI ($p < 0.01$ vs. NPE). Offspring of poly(I:C)-treated mothers also exhibit a deficit in PPI ($p < 0.001$), while the

PPI of offspring of mice injected with poly(I:C) + anti-IL-6 antibody is not significantly different from controls, but it is significantly higher than the PPI of offspring of mice injected with poly(I:C) alone ($p < 0.05$) (Fig. 2b). Thus, a brief neutralization of IL-6 during a critical period of embryonic development prevents two important behavioral deficits caused by MIA.

Heightened anxiety and deficits in social interaction are hallmarks of schizophrenia and autism. Reluctance to enter the center portion of a well-lit open field is usually taken as a measure of heightened anxiety under mildly stressful conditions (Shi et al., 2003). Compared to controls, the offspring of poly(I:C)-injected mice exhibit a deficit in open field exploration, as measured by entries into the center of the field ($p < 0.05$) or by total distance traversed ($p < 0.01$) (Fig. 2c,d). Neutralization of IL-6 prevents these deficits ($p < 0.05$ and 0.001, respectively). In the social interaction assay, control mice spend more time in the social chamber of the apparatus compared to the non-social chamber, while offspring of poly(I:C)-injected mice do not show a preference for the social chamber ($p < 0.05$) (Fig. 2f). Once again, neutralization of IL-6 prevents this deficit ($p < 0.05$).

Effects of MIA in IL-6 KO mice. We also used genetically altered mice to test the requirement for IL-6 in mediating MIA. The adult offspring of pregnant IL-6 KO mice injected with poly(I:C) on E12.5 display robust PPI, similar to that found in adult offspring of saline-injected IL-6 KO mice (control 73.0 ± 5.9 %; poly(I:C) 81.2 ± 2.0 %, $p = 0.15$). Moreover, the groups are similarly active in the open field test (center entries: control 12.7 ± 1.3; poly(I:C) 10.2 ± 1.9, $p = 0.62$; distance traveled: control 3360 ± 201.8 cm; poly(I:C) 3202 ± 243.5 cm $p = 0.30$), and both groups display a similar strong

preference for the social chamber in the three-chamber social interaction test (control 43.5 ± 4.6 %; poly(I:C) 33.2 ± 6.7 %, $p = 0.22$). In the LI paradigm, NPE offspring of both control- and poly(I:C)-injected females do not display any significant conditioning behavior (freezing; data not shown), making LI testing impossible in IL-6 KO mice. The abnormal conditioning behavior of the IL-6 KO mice suggests that IL-6 is important in brain development and/or function, as indicated by other behavioral studies (Armario et al., 1998; Butterweck et al., 2003; Braida et al., 2004). The key point in the present context is, however, that maternal poly(I:C) treatment has no effect on PPI, social interaction or open field behavior of the adult offspring, further demonstrating the importance of IL-6 in causing behavioral deficits in the offspring of MIA mice.

Effect of anti-IL-6 on changes in gene expression evoked by MIA. Another measure of how MIA affects fetal brain development is the alteration in gene expression in the brains of the offspring (Fatemi et al., 2005). Rather than focusing on the identification of individual genes, we used the microarray analysis to asses the overall changes to the transcriptome caused by IL-6 and MIA. mRNA was extracted from frontal cortex, which roughly corresponds to the human prefrontal cortex, an area that shows molecular, functional and microanatomical alterations in human cognitive disorders (Lewis and Levitt, 2002). Tissue from five adult animals from control, poly(I:C), and poly(I:C) + anti-IL-6 groups was individually processed and hybridized. Using a cutoff value of $p<0.01$, 61 significant changes in gene expression are identified in the offspring of the poly(I:C)-treated mice when compared to the saline-treated control animals. Fifty-five of these 61 genes (90%) do not show a statistically significant expression difference when comparing poly(I:C) + anti-IL-6-treated vs. saline-treated animals. Using a two-

way (samples and genes) unsupervised hierarchical clustering of the gene expression intensities, the three experimental groups separate into distinct clusters (Fig. 3). Remarkably, four out of the five mice in the poly(I:C) + anti-IL-6 group cluster with the control group, rather than with the poly(I:C) group. A detailed analysis of the microarray results, including confirmation of changes by PCR and a detailed discussion of the genes identified, will be the subject of a future publication. The point of the analysis presented here is the demonstration that maternal anti-IL-6 treatment prevents the gene expression, as well as behavioral, changes caused by poly(I:C) MIA.

Discussion

Maternal infection is an environmental risk factor for both schizophrenia and autism (Brown and Susser, 2002; Patterson, 2002). The lack of evidence for direct infection of the fetus ((Shi et al., 2005); but see (Aronsson et al., 2002)), and the fact that multiple pathogens cause similar results in humans (e.g., influenza (Brown, 2006), herpes (Babulas et al., 2006), rubella (Chess, 1977)) indicates that MIA in general is detrimental to the developing brain. The data presented here confirms previous reports (see Introduction) that MIA causes behavioral and gene-expression changes in the offspring of pregnant mice. When IL-6 is eliminated from the maternal immune response using genetic methods or with blocking antibodies, however, the behavioral deficits associated with MIA are not present in the adult offspring. Antibodies to IL-1β or IFNγ did not prevent behavioral deficits, suggesting that the anti-IL-6 effect is specific. Furthermore, maternal exposure to IL-6, in the absence of poly(I:C) or infection, is sufficient to cause two key deficits in the adult offspring, PPI and LI disruption. Thus, IL-6 is central to the process by which MIA causes long-term behavioral alterations in the offspring.

We further show that blocking IL-6 eliminates virtually all of the transcriptional changes caused by MIA. The microarray data presented here is essentially numerical data that estimates the extent to which treatment with the anti-IL-6 antibody is able to normalize changes in expression. Our sole aim in including the numerical data here is to demonstrate that blocking IL-6 prevents >90% of the changes seen in offspring of poly(I:C)-injected females, showing that gene expression changes, as well as behavioral changes, are normalized by eliminating IL-6 from the maternal immune response.

Poly(I:C) signals through toll-like receptor 3 (TLR3) via an NFκB-dependent mechanism (Alexopoulou et al., 2001). While usually cited for its ability to induce interferons (Toth et al., 1990; Katafuchi et al., 2003; Voss et al., 2006), poly(I:C) is also a strong inducer of IL-1, IL-6 and TNF-α (Fortier et al., 2004b; Traynor et al., 2004; Gilmore et al., 2005). Our pilot data suggesting that IL-1α, TNF-α and IFN-γ do not cause behavioral changes in the offspring may be surprising, as these cytokines induce IL-6 *in vivo* (Gadient and Otten, 1997). However, high levels of maternal IL-6 are necessary for the behavioral changes we observe in the adult offspring, and it may be that these cytokines (as well as lower i.p. doses of IL-6 or poly(I:C)) do not produce levels of IL-6 sufficient to have an effect on the fetus.

While it is remarkable that a single I.P. injection of IL-6 is capable of altering the fetal brain, leading to abnormal adult behavior, the cytokine stimulation by poly(I:C) is also quite transient. Poly(I:C)-treated mice display sickness behavior (lethargy, hunched posture, hindlimb stiffness) beginning ~30 min after injection and lasting about 6 hours (data not shown), and a biphasic temperature response consisting of 4-8 hours of hyperthermia followed by 12-24 hours of hypothermia (Traynor et al., 2004;

Cunningham et al., 2007). However, the effects of poly(I:C) treatment are mild compared to experimental influenza infection, where sickness behavior and hypothermia last for several days (Yang and Evans, 1961; Shi et al., 2003). Previous work with pregnant rats demonstrated that three injections of IL-6 over five days caused increased latency to find the platform in the Morris water maze in the adult offspring (Samuelsson et al., 2006). The total amount of IL-6 administered to the rats was similar to the total amount administered in our study, although the rats received the dose spread over 5 days. The offspring of the IL-6-injected rats also exhibited pyknotic cells similar to those reported in the offspring of rats injected with poly(I:C) (Zuckerman et al., 2003). This pathology is not, however, observed in the offspring of maternal poly(I:C)-treated mice ((Meyer et al., 2006) and our data, not shown). Early postnatal administration of epidermal growth factor or leukemia inhibitory factor over several days also cause PPI deficits, tested in adulthood (Futamura et al., 2003; Watanabe et al., 2004), but in none of these previous studies were the roles of endogenous cytokines examined.

Ideally, one would like to extend these cytokine-blocking experiments to the model of maternal infection with influenza virus (Shi et al., 2003), as this model more closely recapitulates the human data related to mental illness. However, when pregnant C57 females are infected with influenza virus and given an anti-IL-6 injection, they become more severely ill than mice given saline injections, and either die or suffer miscarriage (data not shown). Similar results were obtained with influenza infection of IL-6 KO mice (data not shown). A previous study on experimental influenza infection in non-pregnant IL-6 KO mice reported changes in weight loss, body temperature and anorexia, but did not report effects on survival (Kozak et al., 1997). That study also only

lasted for 5 days, and we do not see major differences until 6-7 days, when wild-type mice begin to recover and KO mice do not. The increased severity of infection in IL-6-compromised mice forced us to use the poly(I:C), pathogen-free MIA model for the IL-6 blocking experiments.

The observation that elimination of IL-6 in the MIA model almost completely abrogates abnormal behaviors and transcriptome changes in the offspring suggests that searches for other mediators of MIA should be directed up- and down-stream of IL-6, rather than in other signaling pathways. IL-6 is a pleiotropic cytokine that signals through heterodimerization of gp130 and IL-6 receptor (IL-6R) on the cell surface (Bauer et al., 2007). However, most cells express gp130, and the soluble form of the IL-6R that is present in blood allows *trans* signaling (McLoughlin et al., 2005), which enables many cells to respond to IL-6. In considering the location of IL-6 action in the MIA model, three major sites of signaling seem likely: the maternal immune system, the maternal/fetal interface (i.e. the placenta), and the fetal brain.

The most obvious possibility is that IL-6 acts directly on the fetal brain. IL-6 is known to play a role in brain development, learning and memory and in the CNS response to disease and injury (Bauer et al., 2007). IL-6 is central to inflammation-induced working memory disruption (Sparkman et al., 2006) and plays an important role in long-term potentiation in normal rats (Balschun et al., 2004). During development, the STAT pathway, through which IL-6 signals, regulates the balance between neurogenesis and gliogenesis (He et al., 2005) and IL-6 triggers brain endothelial cells to divide and migrate (Yao et al., 2006). After injury, IL-6 can assume very different roles, triggering either neuronal survival or neuronal degeneration, through mechanisms that are not well

understood (Gadient and Otten, 1994; Wagner, 1996; Harry et al., 2006). Regarding access to the fetus, radiolabeled IL-6 can enter the rat fetus during mid, but not late, gestation (Dahlgren et al., 2006), which correlates with human data showing influenza infection increases risk for schizophrenia only in the second trimester (Brown, 2006). Elevated levels of cytokine protein and mRNA (including IL-6) have been detected in embryonic serum and brain after MIA (Fidel et al., 1994; Cai et al., 2000; Urakubo et al., 2001; Gayle et al., 2004; Paintlia et al., 2004; Gilmore et al., 2005; Ashdown et al., 2006; Beloosesky et al., 2006; Meyer et al., 2006; Xu et al., 2006). IL-6 can regulate brain-derived neurotrophic factor (BDNF) expression (Murphy et al., 2000), and a decrease in BDNF is found in embryos and placentas 24 hours after poly(I:C) administration (Gilmore et al., 2005). Thus, while the pleiotropic nature of IL-6 makes it difficult to predict the precise mechanism of action in the brain, many plausible pathways exist.

A second target of interest is the placenta, because IL-6 could alter the transfer of nutrients, hormones or other key molecules to the fetus. IL-6 alters vascular permeability in the adult brain after bacterial challenge (Paul et al., 2003), and expression of genes responsible for the integrity of the placental barrier are decreased after MIA (Beall et al., 2005). This could have significant effects on transfer of potentially harmful proteins (i.e. antibodies) into the fetal environment, or could allow maternal immune cells to infiltrate the fetus.

IL-6 could also act on the maternal immune system, activating lymphocyte migration and cytotoxicity and degrading maternal tolerance of the fetus. Normal pregnancy is characterized by a shift in basal cytokine production and other adjustments to prevent rejection of the fetus (Sargent et al., 2006). Severe MIA causes loss of

pregnancy in rodents, and depletion of uterine natural killer (uNK) cells prevents this loss, indicating that uNK cells mediate the effect (Arad et al., 2005). IL-6 could also enhance production of maternal antibodies, which could cross-react with the fetal brain, as has been proposed to occur in autism (Warren et al., 1990; Dalton et al., 2003; Vincent et al., 2003; Singer et al., 2006; Zimmerman et al., 2007). Future research on the mechanism of MIA effects on fetal brain development and on potential therapeutic approaches can therefore productively focus on the effects of IL-6 on the maternal-fetal unit.

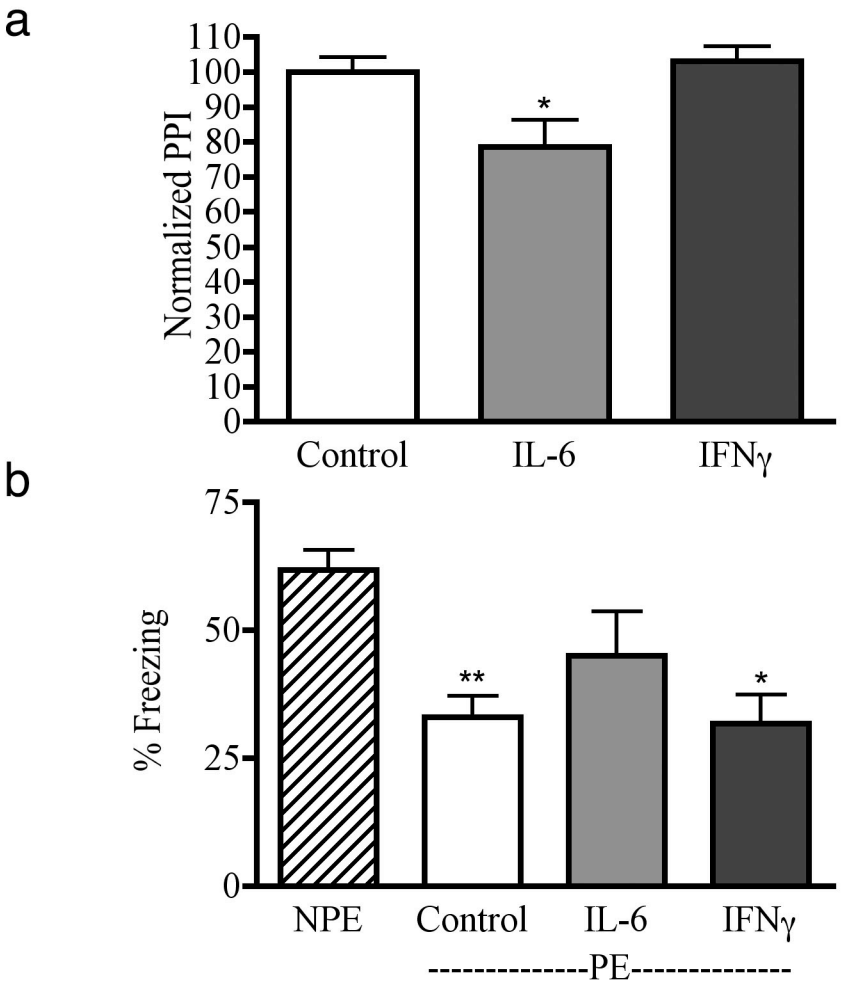


Figure 1. **The offspring of mice injected with recombinant IL-6 exhibit abnormal behaviors**. (a) Offspring of mice injected with IL-6, but not with IFNγ, have a PPI deficit at a prepulse intensity of 85 db. [$F_{2,79}$=4.369, $p < 0.05$] * $p < 0.05$ vs. Control. (b) Pre-exposed (PE) offspring of control mice show normal latent inhibition when compared to not-pre-exposed (NPE) animals, as do PE offspring of IFNγ-injected mothers. Offspring

of IL-6-injected mothers, in contrast, do not demonstrate significant latent inhibition. [$F_{3,103}$=10.22, $p < 0.0001$] * $p < 0.05$ vs NPE; ** $p < 0.001$ vs NPE.

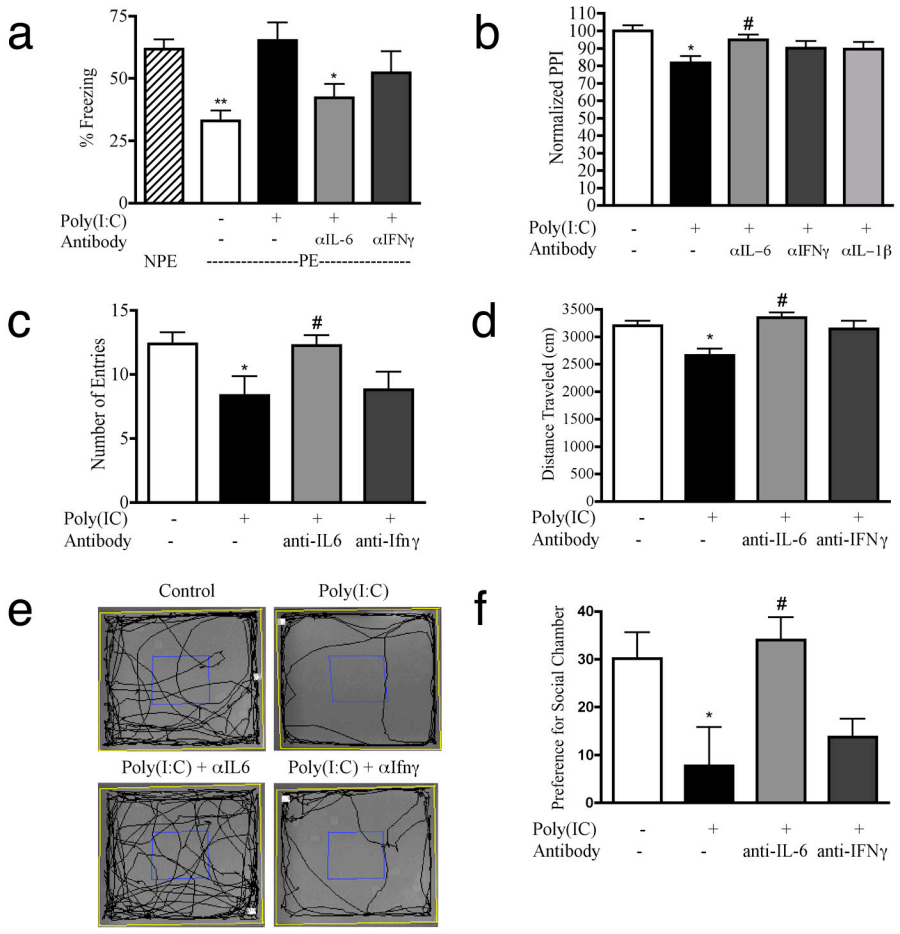


Figure 2: **Abnormal behavior in MIA offspring is prevented by maternal treatment with anti-IL-6 antibody**. (a) Offspring of mice treated with poly(I:C) lack LI. Co-injection of anti-IL-6 with poly(I:C) restores significant LI, while co-injection of anti-

IFNg does not. [$F_{4,132}$=7.566, $p < 0.0001$] ** $p < 0.001$ vs. NPE; * $p < 0.01$ vs. NPE . (b) Compared to controls, the offspring of mice treated with poly(I:C) show a PPI deficit at a prepulse level of 85 db. Co-injection with anti-IL6 prevents this deficit. The PPI of offspring of mice co-injected with poly(I:C) and anti-IFNγ or anti-IL-1b are not significantly different from control or poly(I:C). [$F_{4,270}$=4.195, $p < 0.005$]; * $p < 0.001$ vs. control; # $p < 0.05$ vs poly(I:C). In the open field test, offspring of mice treated with poly(I:C) make fewer entries than controls into the center (c) and travel less total distance (d). Offspring of mice co-injected with anti-IL-6 enter the center as often as control mice [$F_{3,123}$=3.703, $p<0.05$]; * $p < 0.05$ vs. control, # $p < 0.05$ vs. poly(I:C), and move a similar total distance [$F_{3,123}$=6.666, $p < 0.0005$]; * $p < 0.01$ vs. control; # $p < 0.001$ vs. poly(I:C). Offspring of mice co-injected with poly(I:C) and anti-IFNγ are not significantly different from controls or poly(I:C). (e) Tracks recorded during the open field session demonstrate increased thigmotaxis in offspring of poly(I:C)-treated mice compared to offspring of mice co-injected with an IL-6 antibody. (f) In the social interaction test, control mice show a strong preference for the social chamber (defined as (percent time in social chamber) – (percent time in opposite chamber)), while the offspring of poly(I:C)-treated mice show no such preference. Again, the deficit is corrected by maternal administration of IL-6 antibody. [$F_{3,50}$=4.244; $p < 0.01$]; * $p < 0.05$ vs control; # $p < 0.05$ vs poly(I:C).

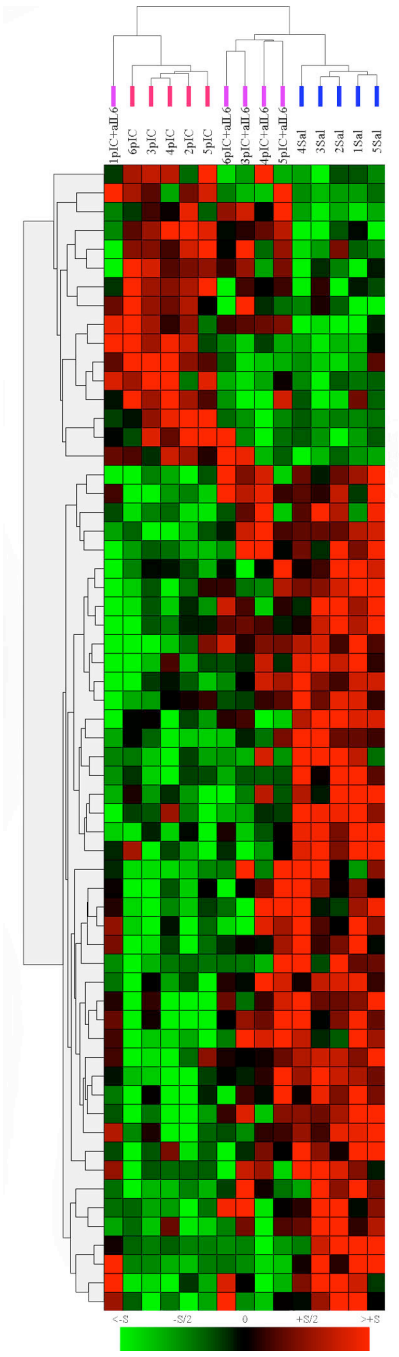


Figure 3: Unsupervised clustering of microarray data shows anti-IL6 treatment rescues transcriptome changes in MIA offspring. Sixty one genes show significant ($p<.01$) expression differences between the adult offspring of poly(I:C)-treated and PBS-

injected mice. Two-dimensional, unsupervised clustering of these genes (X axis-genes; Y axis-samples) reveals control (SAL, blue bars), poly(I:C) (PIC, red bars) and poly(I:C) + anti-IL-6 (PIC + aIL6, purple bars) animals cluster according to treatment, with only one PIC+aIL6 outlier. Significantly, the PIC + aIL-6 animals cluster with PBS-injected controls, rather than with PIC offspring. Each column represents expression values from a single animal. Genes are annotated by Afymetrix probe set/Unigene identifiers. Color intensity represents the magnitude of the gene expression change compared to the overall average intensity (green-decreased; red-increased; black-unchanged).

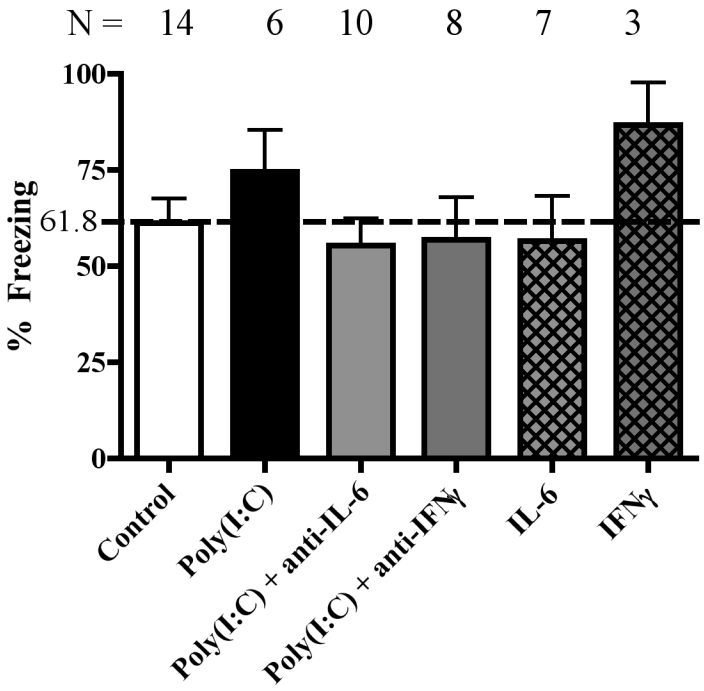
Supplementary Table 1: Numbers of Animals: Table A shows the antibody-blocking experiments and table B shows the cytokine administration and IL-6 KO experiments. The numbers of total offspring and the number of litters (in parenthesis) are shown. Five pregnant females were treated in each group, but not all females produced pups. There were no significant differences by ANOVA in litter size or litter number [Experiment 1: $F(6,17)=1.31$, $p>>.05$, Experiment 2: ANOVA $F(4,16)=1.05$, $p>>.05$]. The loss of litters resulted from spontaneous abortion or cannibalization of young pups, and did not appear related to treatment group.

A)

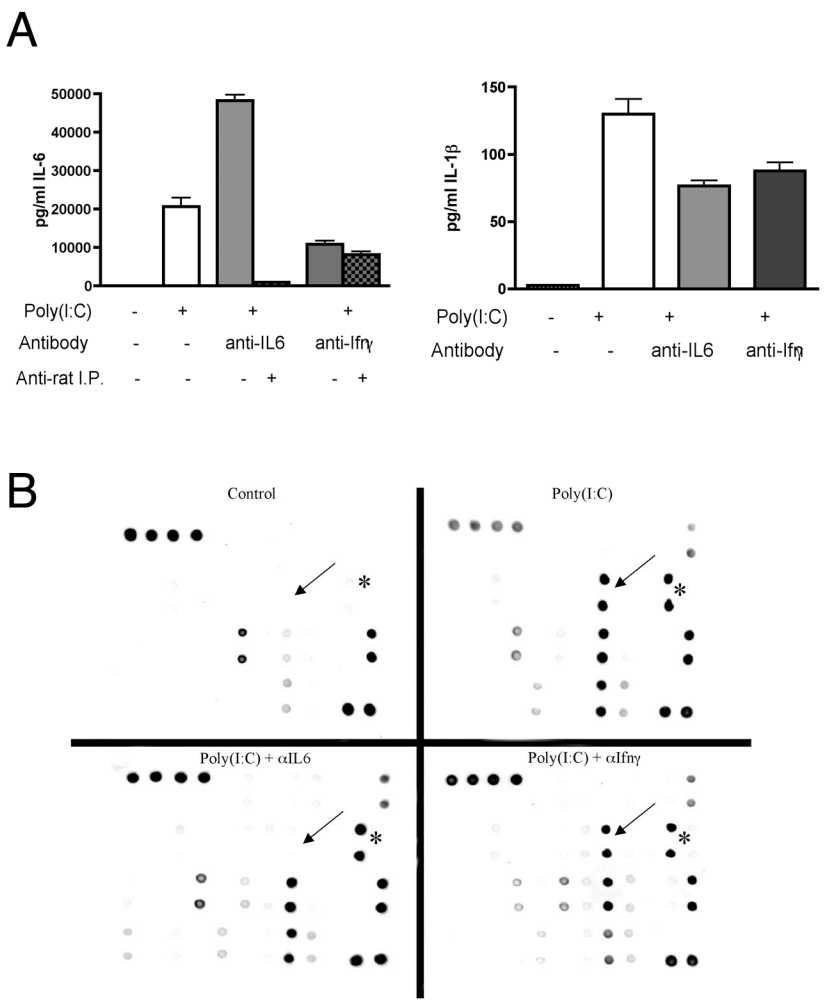
Expt #	*Control*	*Poly(I:C)*	*Poly(I:C) + anti-IL-6*	*Poly(I:C) + anti-IFNg*	*Poly(I:C) + anti-IL-1β*
1	*41 (5)*	*30 (4)*	*19 (3)*	*-*	*39 (5)*
2	*27 (4)*	*22 (3)*	*41 (5)*	*20 (3)*	*-*

B)

IL-6	*IFNγ*	*Anti-IL-6 (antibody control)*	*IL6KO/Control*	*IL6 KO/Poly(I:C)*
20 (3)	*14 (3)*	*21 (3)*	*15 (3)*	*17 (3)*



Supplimentary Figure 1: Conditioned freezing in not-pre-exposed (NPE) animals does not differ among groups. All groups were sub-divided into two sub-groups for latent inhibition testing, PE and NPE. NPE mice did not receive pre-exposure to the tone. When NPE mice were tested for conditioned freezing two days after pairing, there were no differences among the groups [ANOVA $F(5, 42)=1.072$, $p > .05$]. Because of the low numbers of animals in each sub-group, all NPE mice were merged into a single NPE group for greater statistical power. The percent freezing of the merged NPE group is shown by the dotted line.



Supplementary Figure 2: Cytokine levels in response to treatments. (A) IL-6 and IL-1β levels were measured by ELISA. IL-6 levels are elevated in all pregnant mice administered poly(I:C), and are unexpectedly higher in mice co-administered anti-IL6. Magnetic beads coated with anti-rat antiserum were used to immunoprecipitate the administered antibodies, and the ELISA was repeated. More than 98% of IL-6 detected in the serum of mice injected with anti-IL6 is removed after I.P. The higher initial levels of IL-6 in the poly(I:C) + anti-IL-6 group can probably be attributed to stabilization of the cytokine in serum by the antibody. IL-1β levels are not significantly different among

groups. (B) A semi-quantitative cytokine array reveals elevation in the levels of several cytokines, including IL-6 (arrows) and IL-12 (asterisks), in response to poly(I:C) administration. Anti-IL-6 treatment prevents IL-6 detection in this assay, without affecting the levels of other cytokines. Note that IL-6 is not detected in serum from saline- or poly(I:C) + anti-IL-6- injected females. Cytokines represented:

	A	B	C	D	E	F	G	H	I	J	K	L
1	POS	POS	NEG	NEG	Blank	BLC	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
2	POS	POS	NEG	NEG	Blank	BLC	CD30 L	Eotaxin	Eotaxin-2	Fas Ligand	Fractalkine	GCSF
3	GM-CSF	IFN_	IL-1_	IL-1	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
4	GM-CSF	IFN_	IL-1_	IL-1	IL-2	IL-3	IL-4	IL-6	IL-9	IL-10	IL-12p40p70	IL-12p70
5	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1_	MIP-1_
6	IL-13	IL-17	I-TAC	KC	Leptin	LIX	Lymphotactin	MCP-1	MCSF	MIG	MIP-1_	MIP-1_
7	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF_	sTNF R1	sTNF RII	Blank	Blank	POS
8	RANTES	SDF-1	TCA-3	TECK	TIMP-1	TIMP-2	TNF_	sTNF R1	sTNF RII	Blank	Blank	POS

Adapted from http://www.raybiotech.com/map/mouse_inflammation_I_map.pdf

References

Alexopoulou L, Holt AC, Medzhitov R, Flavell RA (2001) Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. Nature 413:732-738.

Arad M, Atzil S, Shakhar K, Adoni A, Ben-Eliyahu S (2005) Poly I-C induces early embryo loss in f344 rats: a potential role for NK cells. Am J Reprod Immunol 54:49-53.

Armario A, Hernandez J, Bluethmann H, Hidalgo J (1998) IL-6 deficiency leads to increased emotionality in mice: evidence in transgenic mice carrying a null mutation for IL-6. J Neuroimmunol 92:160-169.

Aronsson F, Lannebo C, Paucar M, Brask J, Kristensson K, Karlsson H (2002) Persistence of viral RNA in the brain of offspring to mice infected with influenza A/WSN/33 virus during pregnancy. J Neurovirol 8:353-357.

Ashdown H, Dumont Y, Ng M, Poole S, Boksa P, Luheshi GN (2006) The role of cytokines in mediating effects of prenatal infection on the fetus: implications for schizophrenia. Mol Psychiatry 11:47-55.

Babulas V, Factor-Litvak P, Goetz R, Schaefer CA, Brown AS (2006) Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. Am J Psychiatry 163:927-929.

Balschun D, Wetzel W, Del Rey A, Pitossi F, Schneider H, Zuschratter W, Besedovsky HO (2004) Interleukin-6: a cytokine to forget. Faseb J 18:1788-1790.

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. Nat Rev Neurosci 8:221-232.

Beall MH, Amidi F, Gayle DA, Wang S, Beloosesky R, Ross MG (2005) Placental and fetal membrane Nephrin and Neph1 gene expression: response to inflammation. J Soc Gynecol Investig 12:298-302.

Beloosesky R, Gayle DA, Amidi F, Nunez SE, Babu J, Desai M, Ross MG (2006) N-acetyl-cysteine suppresses amniotic fluid and placenta inflammatory cytokine responses to lipopolysaccharide in rats. Am J Obstet Gynecol 194:268-273.

Braida D, Sacerdote P, Panerai AE, Bianchi M, Aloisi AM, Iosue S, Sala M (2004) Cognitive function in young and adult IL (interleukin)-6 deficient mice. Behav Brain Res 153:423-429.

Brown AS (2006) Prenatal infection as a risk factor for schizophrenia. Schizophr Bull 32:200-202.

Brown AS, Susser ES (2002) In utero infection and adult schizophrenia. Ment Retard Dev Disabil Res Rev 8:51-57.

Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP, Susser ES (2004) Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 61:774-780.

Butterweck V, Prinz S, Schwaninger M (2003) The role of interleukin-6 in stress-induced hyperthermia and emotional behaviour in mice. Behav Brain Res 144:49-56.

Cai Z, Pan ZL, Pang Y, Evans OB, Rhodes PG (2000) Cytokine induction in fetal rat brains and brain injury in neonatal rats after maternal lipopolysaccharide administration. Pediatr Res 47:64-72.

Chess S (1977) Follow-up report on autism in congenital rubella. J Autism Child Schizophr 7:69-81.

Cunningham C, Campion S, Teeling J, Felton L, Perry VH (2007) The sickness behaviour and CNS inflammatory mediator profile induced by systemic challenge of mice with synthetic double-stranded RNA (poly I:C). Brain Behav Immun 21:490-502.

Dahlgren J, Samuelsson AM, Jansson T, Holmang A (2006) Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr Res 60:147-151.

Dalton P, Deacon R, Blamire A, Pike M, McKinlay I, Stein J, Styles P, Vincent A (2003) Maternal neuronal antibodies associated with autism and a language disorder. Ann Neurol 53:533-537.

Fatemi SH, Pearce DA, Brooks AI, Sidwell RW (2005) Prenatal viral infection in mouse causes differential expression of genes in brains of mouse progeny: a potential animal model for schizophrenia and autism. Synapse 57:91-99.

Fidel PL, Jr., Romero R, Wolf N, Cutright J, Ramirez M, Araneda H, Cotton DB (1994) Systemic and local cytokine profiles in endotoxin-induced preterm parturition in mice. Am J Obstet Gynecol 170:1467-1475.

Fortier ME, Joober R, Luheshi GN, Boksa P (2004a) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res 38:335-345.

Fortier ME, Kent S, Ashdown H, Poole S, Boksa P, Luheshi GN (2004b) The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism. Am J Physiol Regul Integr Comp Physiol 287:R759-766.

Futamura T, Kakita A, Tohmi M, Sotoyama H, Takahashi H, Nawa H (2003) Neonatal perturbation of neurotrophic signaling results in abnormal sensorimotor gating and social interaction in adults: implication for epidermal growth factor in cognitive development. Mol Psychiatry 8:19-29.

Gadient RA, Otten U (1994) Expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat brain during postnatal development. Brain Res 637:10-14.

Gadient RA, Otten UH (1997) Interleukin-6 (IL-6)--a molecule with both beneficial and destructive potentials. Prog Neurobiol 52:379-390.

Gayle DA, Beloosesky R, Desai M, Amidi F, Nunez SE, Ross MG (2004) Maternal LPS induces cytokines in the amniotic fluid and corticotropin releasing hormone in the fetal rat brain. Am J Physiol Regul Integr Comp Physiol 286:R1024-1029.

Gilmore JH, Jarskog LF, Vadlamudi S (2005) Maternal poly I:C exposure during pregnancy regulates TNF alpha, BDNF, and NGF expression in neonatal brain and the maternal-fetal unit of the rat. J Neuroimmunol 159:106-112.

Harry GJ, Lawler C, Brunssen SH (2006) Maternal infection and white matter toxicity. Neurotoxicology 27:658-670.

He F, Ge W, Martinowich K, Becker-Catania S, Coskun V, Zhu W, Wu H, Castro D, Guillemot F, Fan G, de Vellis J, Sun YE (2005) A positive autoregulatory loop of Jak-STAT signaling controls the onset of astrogliogenesis. Nat Neurosci 8:616-625.

Irizarry RA, Bolstad BM, Collin F, Cope LM, Hobbs B, Speed TP (2003a) Summaries of Affymetrix GeneChip probe level data. Nucleic Acids Res 31:e15.

Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP (2003b) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 4:249-264.

Katafuchi T, Kondo T, Yasaka T, Kubo K, Take S, Yoshimura M (2003) Prolonged effects of polyriboinosinic:polyribocytidylic acid on spontaneous running wheel activity and brain interferon-alpha mRNA in rats: a model for immunologically induced fatigue. Neuroscience 120:837-845.

Kozak W, Poli V, Soszynski D, Conn CA, Leon LR, Kluger MJ (1997) Sickness behavior in mice deficient in interleukin-6 during turpentine abscess and influenza pneumonitis. Am J Physiol 272:R621-630.

Lewis DA, Levitt P (2002) Schizophrenia as a disorder of neurodevelopment. Annu Rev Neurosci 25:409-432.

McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA (2005) IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proc Natl Acad Sci U S A 102:9589-9594.

Medesan C, Cianga P, Mummert M, Stanescu D, Ghetie V, Ward ES (1998) Comparative studies of rat IgG to further delineate the Fc:FcRn interaction site. Eur J Immunol 28:2092-2100.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Murphy PG, Borthwick LA, Altares M, Gauldie J, Kaplan D, Richardson PM (2000) Reciprocal actions of interleukin-6 and brain-derived neurotrophic factor on rat and mouse primary sensory neurons. Eur J Neurosci 12:1891-1899.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Paintlia MK, Paintlia AS, Barbosa E, Singh I, Singh AK (2004) N-acetylcysteine prevents endotoxin-induced degeneration of oligodendrocyte progenitors and hypomyelination in developing rat brain. J Neurosci Res 78:347-361.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Patterson PH (2005) Maternal influenza infection leads to neuropathology and behavioral abnormalities in adult offspring. Neuropsychopharmacology 30:S9-S9.

Paul R, Koedel U, Winkler F, Kieseier BC, Fontana A, Kopf M, Hartung HP, Pfister HW (2003) Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis. Brain 126:1873-1882.

Perry W, Minassian A, Lopez B, Maron L, Lincoln A (2006) Sensorimotor Gating Deficits in Adults with Autism. Biol Psychiatry.

Samuelsson AM, Jennische E, Hansson HA, Holmang A (2006) Prenatal exposure to interleukin-6 results in inflammatory neurodegeneration in hippocampus with NMDA/GABA(A) dysregulation and impaired spatial learning. Am J Physiol Regul Integr Comp Physiol 290:R1345-1356.

Sankoorikal GM, Kaercher KA, Boon CJ, Lee JK, Brodkin ES (2006) A mouse model system for genetic analysis of sociability: C57BL/6J versus BALB/cJ inbred mouse strains. Biol Psychiatry 59:415-423.

Sargent IL, Borzychowski AM, Redman CW (2006) NK cells and human pregnancy--an inflammatory view. Trends Immunol 27:399-404.

Shi L, Tu N, Patterson PH (2005) Maternal influenza infection is likely to alter fetal brain development indirectly: the virus is not detected in the fetus. Int J Dev Neurosci 23:299-305.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 23:297-302.

Singer HS, Morris CM, Williams PN, Yoon DY, Hong JJ, Zimmerman AW (2006) Antibrain antibodies in children with autism and their unaffected siblings. J Neuroimmunol 178:149-155.

Sparkman NL, Buchanan JB, Heyen JR, Chen J, Beverly JL, Johnson RW (2006) Interleukin-6 facilitates lipopolysaccharide-induced disruption in working memory and expression of other proinflammatory cytokines in hippocampal neuronal cell layers. J Neurosci 26:10709-10716.

Tochigi M, Okazaki Y, Kato N, Sasaki T (2004) What causes seasonality of birth in schizophrenia? Neurosci Res 48:1-11.

Toth FD, Juhl C, Norskov-Lauritsen N, Mosborg Petersen P, Ebbesen P (1990) Interferon production by cultured human trophoblast induced with double stranded polyribonucleotide. J Reprod Immunol 17:217-227.

Traynor TR, Majde JA, Bohnet SG, Krueger JM (2004) Intratracheal double-stranded RNA plus interferon-gamma: a model for analysis of the acute phase response to respiratory viral infections. Life Sci 74:2563-2576.

Urakubo A, Jarskog LF, Lieberman JA, Gilmore JH (2001) Prenatal exposure to maternal infection alters cytokine expression in the placenta, amniotic fluid, and fetal brain. Schizophr Res 47:27-36.

Vincent A, Dalton P, Clover L, Palace J, Lang B (2003) Antibodies to neuronal targets in neurological and psychiatric diseases. Ann N Y Acad Sci 992:48-55.

Voss T, Rummel C, Gerstberger R, Hubschle T, Roth J (2006) Fever and circulating cytokines induced by double-stranded RNA in guinea pigs: dependence on the route of administration and effects of repeated injections. Acta Physiol (Oxf) 187:379-389.

Wagner JA (1996) Is IL-6 both a cytokine and a neurotrophic factor? J Exp Med 183:2417-2419.

Warren RP, Cole P, Odell JD, Pingree CB, Warren WL, White E, Yonk J, Singh VK (1990) Detection of maternal antibodies in infantile autism. J Am Acad Child Adolesc Psychiatry 29:873-877.

Watanabe Y, Hashimoto S, Kakita A, Takahashi H, Ko J, Mizuno M, Someya T, Patterson PH, Nawa H (2004) Neonatal impact of leukemia inhibitory factor on neurobehavioral development in rats. Neurosci Res 48:345-353.

Weiner I (2003) The "two-headed" latent inhibition model of schizophrenia: modeling positive and negative symptoms and their treatment. Psychopharmacology (Berl) 169:257-297.

Wynn JK, Dawson ME, Schell AM, McGee M, Salveson D, Green MF (2004) Prepulse facilitation and prepulse inhibition in schizophrenia patients and their unaffected siblings. Biol Psychiatry 55:518-523.

Xu DX, Chen YH, Wang H, Zhao L, Wang JP, Wei W (2006) Tumor necrosis factor alpha partially contributes to lipopolysaccharide-induced intra-uterine fetal growth restriction and skeletal development retardation in mice. Toxicol Lett 163:20-29.

Yang YT, Evans CA (1961) Hypothermia in mice due to influenza virus infection. Proc Soc Exp Biol Med 108:776-780.

Yao JS, Zhai W, Young WL, Yang GY (2006) Interleukin-6 triggers human cerebral endothelial cells proliferation and migration: the role for KDR and MMP-9. Biochem Biophys Res Commun 342:1396-1404.

Zimmerman AW, Connors SL, Matteson KJ, Lee LC, Singer HS, Castaneda JA, Pearce DA (2007) Maternal antibrain antibodies in autism. Brain Behav Immun 21:351-357.

Zuckerman L, Weiner I (2005) Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. J Psychiatr Res 39:311-323.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Chapter 4

Activation of the Maternal Immune System Alters Cerebellar Development in the Offspring

Limin Shi, Stephen E. P. Smith, Natalia Malkova, Doris Tse and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

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Abstract

A common pathological finding in autism is a localized deficit in Purkinje cells (PCs), and cerebellar abnormalities have also been reported in schizophrenia. Using a mouse model that exploits a known risk factor for these disorders, maternal infection, we asked if the offspring of pregnant mice given a mid-gestation respiratory infection have cerebellar pathology resembling that seen in autism. We also tested the effects of maternal immune activation in the absence of virus by injection of the synthetic dsRNA, poly(I:C). We infected pregnant mice with influenza on embryonic day 9.5 (E9.5), or injected i.p. with 20 mg/kg poly(I:C) on E12.5, and assessed the linear density of PCs in the cerebellum of adult or postnatal day 11 (P11) animals. To study granule cell migration, we also injected BrdU on P11, and stained for BrdU at P11, 15 and 17. Adult offspring of influenza- or poly(I:C)-exposed mice display a localized deficit in PCs in lobule VII of the cerebellum, as do P11 animals. Coincident with this are heterotopic PCs, as well as delayed migration of granule cells in lobules VI and VII. The cerebellar pathology observed in the offspring of influenza- or poly(I:C)-exposed mice is strikingly similar to that observed in autism. The poly(I:C) findings indicate that deficits are likely caused by the activation of the maternal immune system. Finally, our data suggest that cerebellar abnormalities occur during embryonic development, and may be an early deficit in autism.

Introduction

Maternal infection can increase the risk in the offspring for mental disorders such as schizophrenia (Brown 2006; Brown 2002; Mednick 1988). The most direct evidence for this comes from a prospective study of pregnant women with medically documented respiratory infections, where the risk for schizophrenia in the offspring is increased 3-fold by infection in the second trimester (Brown 2006). Moreover, the presence of anti-flu antibodies in maternal serum in the first half of pregnancy is associated with a 3-7-fold increase in risk, and elevated levels of the cytokine interleukin-8 in maternal serum is also associated with increased risk for schizophrenia in the offspring (Brown 2004a; Brown 2004b). Although the epidemiology is much less extensive for autism, a >200-fold increase in autism incidence was found in the offspring of maternal rubella infection cases (Chess 1977). While rubella infections can also involve the fetus, smaller studies of other maternal viral infections support the idea that this can be a risk factor for autism (Ciaranello 1995; Hyman 2006). These findings are remarkable given the strong genetic contribution to autism and schizophrenia, which, at present, cannot be controlled for in such epidemiological studies. If it were possible to confine epidemiological analysis to just those individuals with the appropriate susceptibility genotype, the figures for risk cited above could be much higher.

The offspring of mothers with infections display diverse neuropathology, depending in part on the nature and timing of the infection. Following late pregnancy intrauterine bacterial infections, cortical malformation and white matter damage is often seen in the offspring, leading to severe behavioral changes (Dammann 2002; Hagberg 2002). While pathology is more subtle and variable in mental disorders, several reproducible changes

have been identified. A common finding in autism is a localized loss of cerebellar PCs. In 8 studies involving 29 postmortem brains, 72% of the autism cases displayed such a deficit, and structural magnetic resonance imaging (MRI) results support this finding (Palmen 2004). Although there are also negative findings, this is remarkable consistency, particularly in light of the broad spectrum of ages, severity of symptoms, and diversity of autism phenotypes included in these studies.

There is also a very strong inverse correlation between the magnitude of cerebellar lobule VI and VII hypoplasia and the degree of novel object exploration and stereotyped behavior in autistic children (Akshoomoff 2004; Pierce 2001). Moreover, functional MRI reveals abnormal cerebellar activation during motor and cognitive tasks in autistic subjects (Allen 2003; Kates 2004), and there are behavioral abnormalities in autism, such as eye blink conditioning deficits and abnormal visual saccades, which are particularly relevant for the known functions of lobules VI and VII (Nowinski 2005; Takarae 2004). There are, however, reports of negative findings regarding the cerebellum and autism (Kaufmann 2003; Palmen 2004).

There is also evidence for cerebellar pathology in schizophrenia, including PC deficits and reduced cerebellar volume, as well as behavioral evidence (saccades and eye blink conditioning) that points to pathology in lobules VI and VII (Bottmer and Schroder 2005; Brown and O'Donnell 2005; Ho 2004). The identification of cerebellar pathology in these disorders supports an increasing recognition of a role for the cerebellum in higher functions such as cognition, language and learning (Allen 2006; Ramnani 2001; Schmahmann 2001; Schutter 2005; Shi 2003).

In investigating the effects of maternal respiratory infection on fetal brain development, we found that adult offspring of pregnant mice given intranasal influenza on embryonic day 9.5 (E9.5) exhibit behavioral abnormalities reminiscent of autism and schizophrenia, some of which can be ameliorated by anti-psychotic drug treatment (Shi 2003). Many of these behaviors can also be evoked in the absence of viral infection, by activating the maternal immune response with synthetic dsRNA (poly(I:C)) injection (Shi 2003). We and Fatemi et al. found that the offspring of influenza-infected mice display histopathology consistent with that seen in schizophrenia, such as thinning of the neocortex and hippocampus, pyramidal cell atrophy, reduced levels of reelin immunoreactivity, and changes in the expression of neuronal nitric oxide synthase (NOS) and synaptosome associated protein of 25 kD (SNAP25) (Fatemi and Sidwell 2002; Fatemi and Sheikh 1999; Fatemi 2005). Additional neuropathology and behavioral abnormalities have been subsequently reported in the poly (I:C) model of maternal immune activation (Meyer U et al 2005; Meyer 2006; Ozawa et al 2006; Zuckerman 2003; Zuckerman 2005), although no comments were made concerning cerebellar pathology. Using both the influenza-infection and poly(I:C) models, we here report a localized histopathology in the cerebellum that is very similar to that seen in autism and schizophrenia.

Materials and Methods

*Viral infection***.** All procedures involving animals were approved by the Caltech Animal Care and Use Committee. The Balb/C mice were obtained from Simonson Laboratories (Gilroy, CA) and C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, ME). Both strains were bred in the Caltech animal facility for several generations before use. Human influenza virus, strain A/NWS/33CHINI, was collected

from the supernatant of infected MDCK cells, titered, and stored at -80° until use. Pregnant Balb/C mice were anesthetized intra-peritoneally (i.p.) with 10 mg/kg xylazine and 100 mg/kg ketamine on E9.5 (plugged day is day 0) and inoculated intra-nasally with either 6000 plaque-forming units (PFU) of human influenza virus in 90 ml phosphate buffered saline (PBS), or with PBS alone (sham-treatment), or received no treatment (naïve). When no differences were found between the sham and naive groups, the two groups were combined. The number of pups born to control and infected mothers averaged 8 and 4, respectively. The first group of offspring (4 from infected, 4 from sham-infected, and 4 from naïve mothers; 2 males in each group; all from separate litters) were sacrificed at postnatal day 11 (P11). The second group of offspring (4 litters of infected and 3 litters of control) were injected with BrdU (dissolved at 10 mg/ml in saline and injected at 50 mg/kg) at P11. They were sacrificed either after 0.5 hour (6 from infected and 5 from control mothers), day 15 (P15) (6 from infected and 5 from control), or P17 (6 from infected and 7 from control). The third group of offspring was sacrificed as adults (9-14 months of age). These offspring were weaned at P21, and males and females were caged separately in groups of 2-4. Starting at 6 weeks, they were evaluated in open-field and novel object tests, and in a prepulse inhibition (PPI) assay, as described previously (Shi 2003). We selected 7 mice from infected mothers with PPI and open field deficits (5 females and 2 male) and 6 from control mothers with normal behavior (3 females and 3 males) for histochemical analysis. No sex differences were found in linear density of PCs in either lobule V or VII by two-way ANOVA using treatment and sex as main variables, allowing male and female animals to be combined into a single group.

Poly(I:C) maternal immune activation. C57BL/6J mice were used for these

experiments as they tended to give larger litter sizes than Balb/C mice following treatment. Four pregnant mice were injected i.p. with 20 mg/kg poly(I:C) (Sigma, Cat. P9582) freshly dissolved in 0.9% sterile PBS, in an injection volume of 8 ml/kg, on E12.5. Five control females were injected with the same volume of PBS. The offspring were undisturbed until weaning on P21 (32 control, 24 poly(I:C)). Offspring were behaviorally tested starting at 6 weeks and found to have significant deficits ($p < 0.05$) in latent inhibition (Smith 2007) and open field and novel object behavior and a trend toward significance in the PPI test (Shi 2003). Four control and 4 poly (I:C)-exposed offspring (2 males in each group), each from different litters, were randomly selected for sacrifice at 4 months of age for histology. As with the flu-exposed offspring, no sex differences were found in linear density of PCs in either lobule V or VII by two-way ANOVA, and male and female animals were combined into a single group.

*Immunohistochemistry***.** All mice were perfused intracardially with 4% paraformaldehyde in PBS, and post-fixed in that solution at 4° overnight. Following post-fixation, the brains were transferred to a sucrose gradient (from 10 to 20%) at 4° until the tissue sank to the bottom of the container. The brains were then rapidly frozen using a methylbutane bath in dry ice and were stored at -80° until use. Cryostat sections of the cerebellum vermis were cut at 18 mm for adults and 14 mm for young mice. For calbindin immunostaining, sections were treated with 0.05% sodium citrate buffer and heated to boiling seven times over 30 min in a microwave oven. Sections were then cooled and treated with the M.O.M kit (Vector) to block endogenous mouse IgG, blocked with 10% normal goat or horse serum for 30 min, and incubated overnight at 4° with a 1:200 dilution of mouse monoclonal, anti-calbindin, ascites antibody (Sigma). For BrdU staining, slides

were treated with 2 N HCl in Tris buffer (TBS) and 0.5% Tween for 60 min at 37°, followed by 10 minutes in 0.1 M sodium borate. They were incubated overnight with rat monoclonal, anti-BrdU antibody (Immunologicals.com) in TBS containing 10% normal goat serum, 0.5% Tween at 4°, washed in TBS (pH 8.0) with 0.5% Tween-20 for 20 min at room temperature. For GABA _6 receptor staining, sections were incubated in 10% normal goat serum for 30 min, followed by overnight treatment with rabbit anti-GABA _6 receptor antibody (1:500, Chemcon). For florescent images, AlexaFlour 488- or 568-conjugated, goat anti-mouse or anti-rat (1:200, Molecular Probes) antibodies were applied at RT for 2 hours, followed by several PBS washes and mounting with 50% glycerol. For immuohistochemistry, HRP-conjugated secondary antibody (goat anti-rat, Vector) was applied at RT for 2 hours, followed by several PBS washes. The DAB kit (Vector) was used for detection according to manufacturer's instructions, followed by counterstaining with 0.45% Cresyl Violet, clearing through xylene, and mounting with Permount (Fisher).

*Green NeuroTrace Fluorescent Nissl staining***.** Every fourth section of P11 and P17 cerebellum vermis in the BrdU-injected groups was stained for Green NeuroTrace Fluorescent Nissl Stain (Molecular Probes), which binds to Nissl substance and is present exclusively in the somata of neurons. Sections were treated with 0.1% Triton X-100 in PBS for 10 min, washed in PBS 3 times, incubated with 1:200 NeuroTrace in PBS for 20 min, and washed for 10 min in 0.1% Triton X-100, washed in PBS, and mounted.

Image capture: A Nikon fluorescence microscope was used to observe sections; sections of stained brains were first captured digitally using an RT Slider Spot digital camera (Nikon) at 2.5x, 4x, 10x and 20x magnification. Images were then imported into PhotoShop7.0 (Adobe) or ImageJ (NIH) to analyze. Images that were used for counting

PCs were captured at 10x, and at this magnification we were able to capture the entire thickness of the section in the focal plane, thus ensuring complete counting of PCs without using confocal microscopy.

*Quantitation***.** To ensure exhaustive sampling of the cerebellum, every tenth section in adult, and every fourth section in P11, were stained for calbindin. The linear density of PCs was determined by measuring the length of a line drawn along the PC layer from the middle of the cleft between lobules VI and VII to the cleft between lobules VII and VIII, and counting PCs along this line. In the case of lobule V, a line was drawn along the PC layer in the dorsal half of this very large lobule, and PC density determined along this line. The average linear density for each adult animal was calculated by averaging the linear density from between 7-12 sections covering the entire lobule VII. In P11 cerebella, there was a high variability in the length (size) of the lobules, while the PC number/lobule was relatively constant. Therefore, for this developmental stage we used the total PCs per lobule as the data for comparison between experimental groups.

Every fourth cerebellum vermis section in BrdU-injected animals was stained for BrdU. For the brains collected 0.5 hour after BrdU injection on P11, the linear density of granule cells (GCs) in lobule VII was determined by measuring the length of a line drawn along the outline of the lobule (defined as above), and counting all BrdU+ GCs. For the brains sacrificed at P15 and P17, BrdU+ cells in the molecular layer (ML) were counted.

Every fourth section in P17 brains was stained with Green NeuroTrace fluorescent dye. The thickness of the EGL and ML was measured (ImageJ) at three sites in each lobule.

Statistical analysis. Statistical analysis was performed with Prism software (Graphpad). In all experiments, we averaged data from sections from individual mice. The averages were then used to determine the mean ± SEM for each experimental group, so that the N reported reflects the number of mice used, not the number of sections analyzed. Statistical significance was assessed using Student's T test.

Results

Purkinje cells in adult offspring. Given the common finding of PC deficits in autism, and the evidence for its localization to lobules VI and VII, we analyzed PCs in the adult offspring of influenza-infected Balb/c mothers. Using an anti-calbindin monoclonal antibody, which in the cerebellum selectively binds PCs, we find fewer of these neurons, specifically in lobule VII, compared to controls (Fig. 1). The section shown here is representative of the 33% difference in linear density between the experimental offspring and the offspring of control mothers (Fig. 2a). To illustrate the localized nature of the deficit, we counted PCs in lobule V and find no difference from the control (Fig. 2a). Qualitative examination of other lobules (I-IV and VIII-X) also indicates no difference between experimental and control cerebella (Fig. 1). The cross-sectional size of the lobules was also assessed, using the linear extent from the middle of the cleft between lobules to the middle of the next cleft. Every tenth section was measured through the entire vermis. Comparing the adult offspring of control and infected mothers, no difference was found in the size of lobule VII (1.642 ± 0.052 mm vs. 1.702 ± 0.048 mm, $p = 0.42$).

Purkinje cells in young offspring. To determine if the PC deficit occurs during development or as neurodegeneration in adulthood, we counted PCs at P11, the earliest stage at which these cells can be reliably stained and counted in a linear array. A statistically significant difference between the offspring of control and infected mothers, similar to that found in adults, is seen (Fig. 2b).

*Heterotopic Purkinje cells***.** In further support of the deficit being a developmental phenomenon, we occasionally see heterotopic PCs in adult cerebella of mice born to infected mothers. These large, calbindin^{+} cells are found primarily in lobules VI and VII (Fig. 3), suggesting abnormal migration during development. We also find heterotopic PCs in the P11 offspring of infected mothers. In these younger animals, the heterotopy occurs at a higher frequency than in the adults, in some cases in every 2-3 sections. These findings, plus a lack of evidence of sick or degenerating PCs in the adult, lead us to conclude that the PC deficit largely emerges during early cerebellar development, prior to P11.

Granule cells in young offspring. Considering the known developmental interactions between PCs and GCs, we charted the development of these cells as well. First, we measured the thickness of the molecular layer (ML), a layer in which GCs and PCs form synapses. In adult and P17 animals, there is no difference in the thickness of the ML (adult: control 95.0 ± 23.7 μm, exposed 99.5 ± 10.7 μm; P17: control 75.2 ± 3.2 μm, exposed 84.4 ± 4.8 μm). These data suggest that the PC reduction is not reflected in the size of the mature ML, perhaps due to the surviving PCs forming more synapses per cell to compensate.

During development, GCs are born in the external granular layer (EGL) and migrate through the ML to their final position in the internal granular layer (IGL). On E17, a time when the EGL in controls is disappearing, the EGL is significantly thicker in offspring of infected mothers (Fig. 4 a-d). This effect is most pronounced in lobules VI and VII (control EGL 9.5 ± 0.4 μm, exposed EGL 15.8 ± 1.2 μm, $p < 0.001$), consistent with the localized deficit in PCs. The abnormally persistent EGL is eventually lost, however, as Nissl staining in adult animals reveals the normal absence of an EGL in both control and exposed offspring (Fig. 4 e,f). To determine if the thicker EGL at P17 is due to a migrational delay, BrdU was injected at P11 to label newly generated GCs, and the mice sacrificed at 0.5 hours, P15 or P17. At 0.5 hours after BrdU injection, we find no difference in BrdU+ GCs in the EGL (cells per section: control, 166 ± 1.7; exposed, 174 ± 26.5), indicating normal levels of proliferation. On P15, there are similar numbers of GCs migrating through the ML towards their final position in the IGL (cells per section: control 74 ± 16, exposed 86 ± 21). However, in mice sacrificed at P17, we find significantly more BrdU+ GCs in the ML of lobule VII of exposed mice (cells per section: control 23 ± 3.5, exposed 51 ± 4.3, $p < 0.001$), suggesting a spatially localized migrational delay in exposed animals. In adult animals, however, no GCs are found in the ML (Fig. 4 g,h).

Purkinje cells in adult offspring of poly(I:C)-immune activated mothers. Many of the behavioral abnormalities seen in the offspring of infected mothers, such as deficits in prepulse inhibition of the acoustic startle (PPI), open field exploration and social interaction, can also be found in the offspring of mothers whose immune systems were activated by injection of poly(I:C) rather than by virus (Meyer U et al 2005; Meyer 2006; Ozawa et al 2006; Shi 2003; Smith 2007). Thus, it was of interest to ask if the PC deficit is

also shared between these two animal models. When the cerebella of adult offspring of poly(I:C)-injected C57Bl/6 mothers are compared to controls, a difference in PC linear density is seen in lobule VII (Fig. 5). This 20% deficit is similar to that observed in the offspring of infected mothers, indicating that the mother's inflammatory response is likely mediating the deficit seen in the maternal infection model. No differences in PC density from controls are found in lobules V or VIII (Fig. 5). No difference between control and experimental groups is found in the length of lobule VII (1.89 ± 0.061 vs. 1.92 ± 0.041 mm, $p < 0.66$).

Discussion

Although there is a strong genetic component to schizophrenia and autism, it is clear that certain environmental factors can strongly raise the risk for these disorders. One of the best studied of these environmental risk factors in schizophrenia is maternal infection, particularly respiratory infection (Brown 2006). In autism, a highly significant effect was seen with maternal rubella infection, although increased risk has also been reported for other maternal viral infections (Hyman 2006; Patterson 2002). In modeling this risk factor in rodents, major abnormalities have been reported in the offspring of infected or immune-activated mothers using behavioral assays that are relevant for schizophrenia and autism. These assays include PPI of the acoustic startle, social interaction, behavior in the open field and with a novel object, amphetamine-induced locomotion, and latent inhibition (Borrell J et al 2002; Fortier 2004; Meyer U et al 2005; Meyer 2006; Ozawa et al 2006; Shi 2003; Zuckerman 2003).

Given the construct and face validity of this model for schizophrenia and autism, it was of interest to examine the cerebellum to determine if there is pathology that resembles that seen in these mental disorders. This is particularly important in the context of autism, where cerebellar pathology is a commonly reported histological and imaging abnormality. Our finding of a localized deficit in PCs strikingly resembles that seen in autism both in location and magnitude, with a 25% reduction in PC linear density reported in autism (Ritvo 1986) and 33% (influenza) and 20% (poly(I:C)) reductions reported here. While significant changes in the cerebellum have also been found in schizophrenia, there has been little evidence reported as to whether such changes may be more prominent in particular lobules. A preliminary study did indicate, however, that the volume of lobules VI and VII inversely correlate with positive symptoms and hallucinations in schizophrenia (Pierson 2003).

What is the basis for the discrete, localized deficit in PCs? At 50-80 μm in diameter, PCs are very large neurons. Each cell has over 200,000 synapses, giving the cell an exceptionally high metabolic demand, which predisposes PCs to both excitotoxicity and ischemic death (Kern 2003). While histologically uniform at a superficial level, the cerebellum can be compartmentalized into a variety of patterns based on expression of particular molecules, the spatial effects of mutations, and its connectivity. The PCs in lobules VI and VII are distinctive, and perhaps more vulnerable to developmental insults, for several reasons: (i) These cells express a unique combination of molecular markers, both in the neonate and the adult (Ozol et al 1999; Rogers 1999). (ii) They receive a distinct set of afferent inputs (olivocerebellar, pontocerebellar and cuneiocerebellar mossy fibers), and they project to specific regions

of the fastigial (pursuit eye movements) and interposed nuclei (Armstrong 2001). (iii) They receive unique migrational cues; in the *weaver* mutant mouse (*girk2*), PC progenitors specifically in lobules VI and VII appear not to migrate outward to form a monolayer (Armstrong 2001). (iv) PCs in lobules VI and VII (and in IX and X) preferentially survive following 3-acetylpyridine ablation of the inferior olive in *Shaker* mutant rats (Tolbert 2000). Numerous mutations and toxic insults have been associated with distinct patterns of PC loss and survival (Sarna 2003), some of which are inversely correlated with expression of the neuroprotective protein HSP25/27. Since the pattern of cell loss in each case is specific to the type of insult, the selective loss of PCs in lobule VII in the influenza model is a particularly important parallel with autism.

When we administer influenza virus at E9.5, the peak of the inflammatory response occurs around E12 (Fritz 1999; Swiergiel 1999; Swiergiel 1997a; Swiergiel 1997b), which is also the time at which we administer poly(I:C). This time window of maximal cytokine production corresponds with the timing of PC neurogenesis. Precursor PCs are born during embryonic days E11-E13 in the mouse. Post mitotic PCs migrate radially from the neuroepithelium of the ventricle towards the cortical surface between E13-E17 along radial glial fibers (Hatten 1999; Hatten 1997; Hatten 1995; Miale 1961; Uzman 1960; Yuasa 1991). By the time of birth, all PCs occupy their position between the EGL and IGL. The activated immune system produces many substances, such as cytokines and chemokines, which have the potential to alter the neurogenesis and migration of PCs. Our data suggest that the primary deficit occurs in this early stage, with maternal immune activation resulting in abnormal migration of PC precursors. It is also possible that PC precursor proliferation is affected.

The abnormal GC development that we observe may be secondary to the PC deficit. Granule cell development is dependent on signals from PCs. Sonic hedgehog is produced by PCs and is required for proliferation of GC precursors, and it induces increased migration of GCs from cortical explants *in vitro* (Dahmane 1999). Our data show that at P17, a time when most BrdU+ GCs have migrated to the IGL in controls, there are still GCs remaining in the ML in exposed animals. Furthermore, these animals have a persistent EGL that is most prominent in lobules VI and VII, suggesting that a pool of GCs have yet to migrate. PC deficits have the potential to slow GC migration due to the lack of Shh or other factors normally produced by PCs. However, it seems that GCs eventually do receive the proper migration cues and form the IGL, because we do not find GCs in the ML of adult animals.

There are several possible functional consequences of the PC deficit we observe. Lobules VI and VII are also called the oculomotor vermis, since their function is linked to eye movements. Our finding that offspring of poly(I:C)-treated mice display abnormalities in classical eye blink conditioning (Lee 2007) could be related to the PC deficits reported here. The fact that abnormalities in eye blink conditioning are also found in autism (Sears 1994; Steinmetz 2001) and schizophrenia (Brown and O'Donnell 2005; Marenco 2003; Sears 2000) is a further link between this disorder and the maternal immune activation model. Recent evidence suggests that abnormalities in eye tracking are present in infants with high risk for autism, suggesting that impaired eye tracking or eye contact may play a role in later deficits in social interaction (Merin 2007). Due to the importance of the oculomotor vermis in these behaviors, understanding the mechanisms that lead to PC deficits could be crucial in understanding the pathology of autism.

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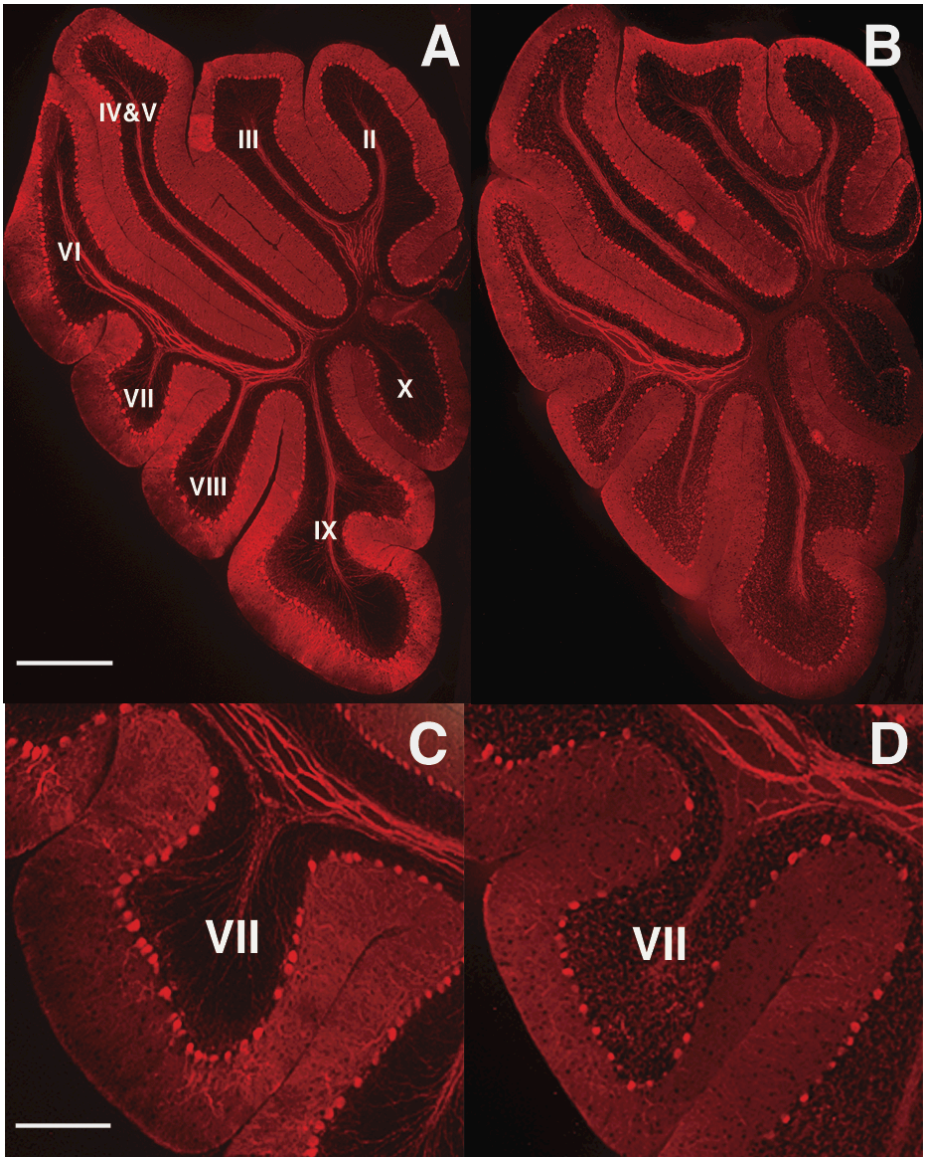

Figure 1. Adult offspring of infected mothers display a PC deficit in lobule VII. Calbindin staining of adult cerebella from offspring of control (A, C) and infected mothers (B, D) reveals a deficit in lobule VII in the latter. Panels C and D (bar = 200 mm) are higher magnification views of panels A and B (bar =800 mm).

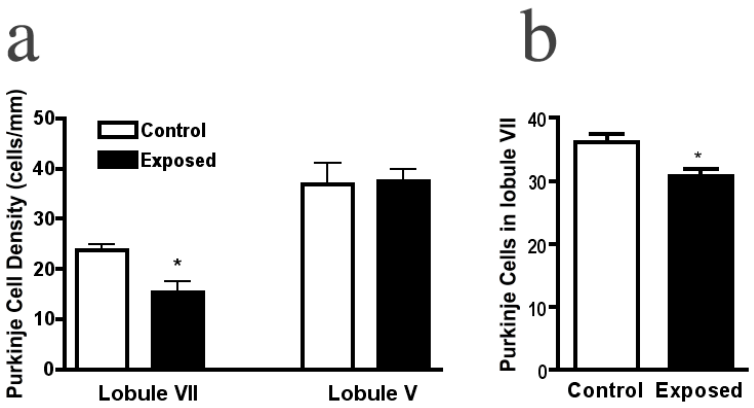

Figure 2. **Young and adult offspring of infected mothers have a PC deficit specifically in lobule VII**. (a) Quantitation of PC linear density reveals a 33% deficit in lobule VII of the adult offspring of infected mothers, while no difference from controls is found in lobule V. (b) A similar, localized deficit is observed in the P11 offspring of infected mothers (* $p < .01$).

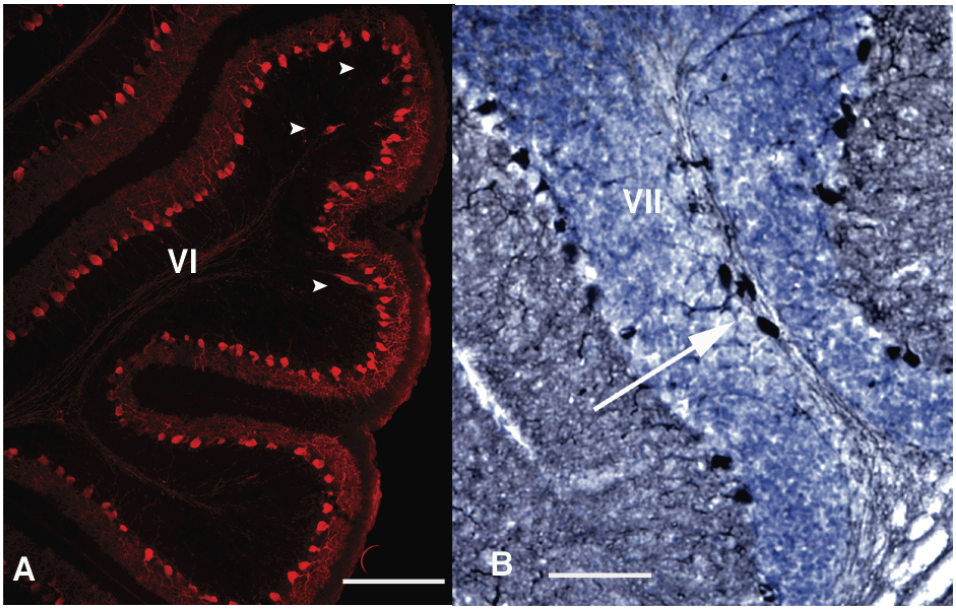

Figure 3. Heterotopic PCs are found in the offspring of infected mothers. Some P11 (A, bar = 200 mm) and adult (B, bar = 100 mm) offspring of infected mothers display large, calbindin+ cells (white arrowheads and arrow) in the white matter of lobules VI or VII. Such cells are rarely seen in other lobules, or in control cerebella.

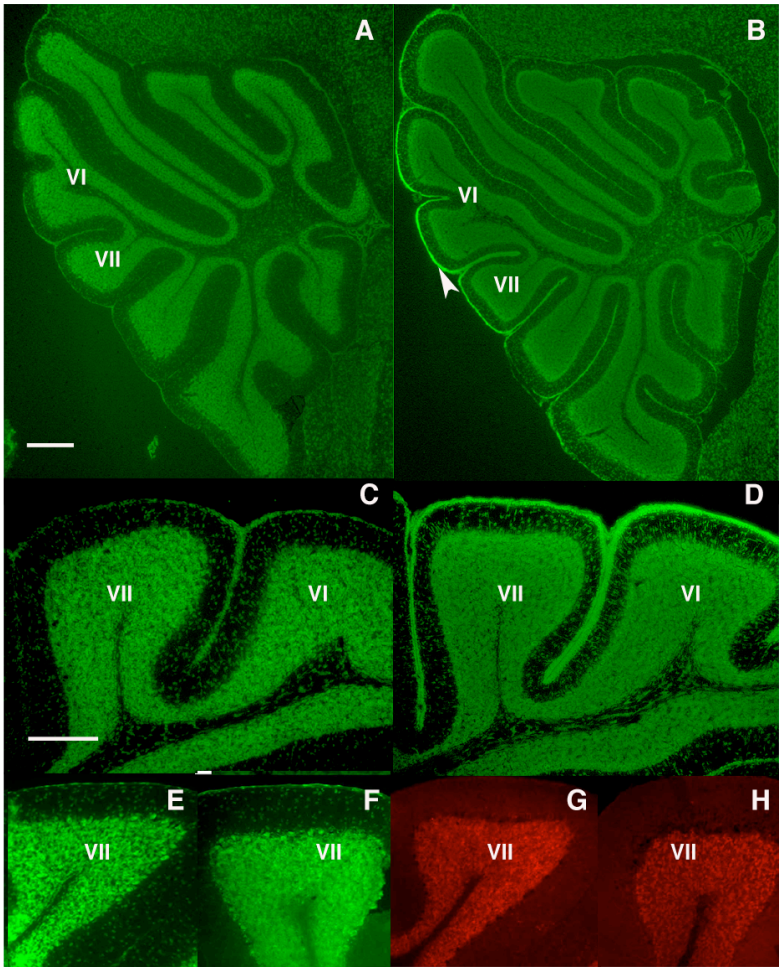

Figure 4. Granule cell development is abnormal in the offspring of infected mothers. At P17, control mice lack an EGL (A, C), while a persistant EGL is observed in the offspring of infected mothers (B, D), particularly around lobules VI and VII (arrowhead). In the adult, Nissl staining reveals the normal absence of an EGL in both control (E) and experimental (F) offspring. Moreover, no GABAR a6 staining is found in the ML of the adult control (G) or experimental (H) offspring, indicating that the GCs have completed their migration into the IGL. Scale bars A, B = 200 mm; C-H = 100 mm.

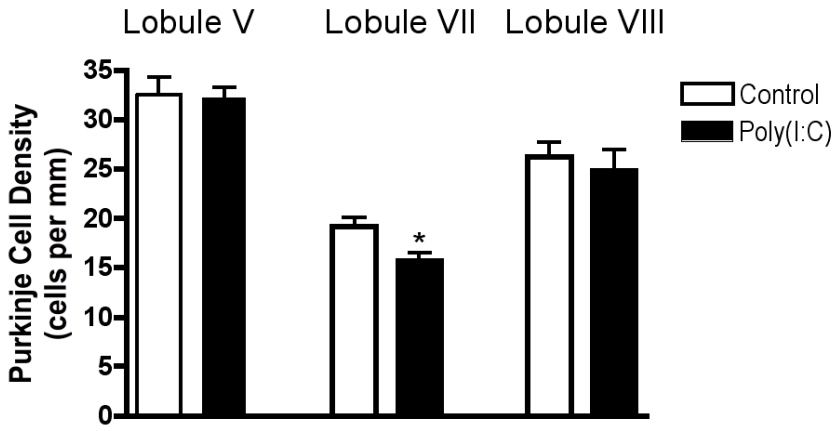

Figure 5. Purkinje cell loss in the adult offspring of immune-activated mothers is localized to lobule VII. A single poly(I:C) injection in pregnant mice causes a deficit in PC density in the adult offspring, specifically in lobule VII. (* $p < 0.02$)

References

Akshoomoff N, Lord, C., Lincoln, A., Courchesne, R., Carper, R., Townsend, J., Courchesne, E. (2004): Outcome classification of preschool children with autism spectrum disorders using MRI brain measures. *Journal of the American Academy Child and Adolescent Psychiatry* 43:349-357.

Allen G (2006): Cerebellar contributions to autism spectrum disorders. *Clinical Neuroscience Research* 6:195-207.

Allen G, Courchesne, E. (2003): Differential effects of developmental cerebellar abnormality on cognitive and motor functions in the cerebellum: an fMRI study of autism. *American Journal of Psychiatry* 160:262-273.

Armstrong C, Hawkes, R. (2001): Selective Purkinje cell ectopia in the cerebellum of the weaver mouse. *Journal of Comparative Neurology* 439:151-161.

Borrell J, Vela JM, Arevalo-Martin A, Molina-Holgado E, C G (2002): Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. *Neuropsychopharmacology* 26:204-215.

Bottmer C, Bachmann, S., Pantel, J., Essig, M., Amann, M., Schad, L.R., Magnotta, V., , Schroder J (2005): Reduced cerebellar volume and neurological soft signs in first-episode schizophrenia. *Psychiatry Research* 140:239-250.

Brown AS (2006): Prenatal infection as a risk factor for schizophrenia. *Schizophrenia Bulletin* 32:200-202.

Brown AS, Begg, M., Gravenstein, S., Schaefer, C., Wyatt, R., Bresnahan, M., Babulas, V., Susser, E. (2004a): Serologic evidence of prenatal influenza in the etiology of schizophrenia. *Archives of General Psychiatry* 161:774-480.

Brown AS, Hooton, J., Schaefer, C., Zhang, H., Petkova, E., Babulas, V., Perrin, M., Gorman, J., Susser, E. (2004b): Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. *American Journal of Psychiatry* 161:889-895.

Brown AS, Susser, E.S. (2002): In utero infection and adult schizophrenia. *Mental Retardation and Developmental Disabilities Research Reviews* 8:51-57.

Brown SM, Kieffaber, P.D., Carroll, C.A., Vohs, J.L., Tracy, J.A., Shekhar, A., , O'Donnell BF, Steinmetz, J.E., Hetrick, W.P. (2005): Eyeblink conditioning deficits indicate timing and cerebellar abnormalities in schizophrenia. *Brain and Cognition* 58:94-108.

Chess S (1977): Follow-up report on autism in congenital rubella. *Journal of Autism and Childhood Schizophrenia* 7:69-81.

Ciaranello AL, Ciaranello, R.D. (1995): The neurobiology of infantile autism. *Annual Review of Neuroscience* 18:101-128.

Dahmane N, Ruiz, I., Altaba, A. (1999): Sonic hedgehog regulates the growth and patterning of the cerebellum

. *Development* 126:3089-3100.

Dammann O, Kuban, K.C., Leviton, A. (2002): Perinatal infection, fetal inflammatory response, white matter damage, and cognitive limitations in children born

preterm. *Mental Retardation and Developmental Disabilities Research Reviews* 8:46-50.

Fatemi SH, Earle, J., Kanodia, R., Kist, D., Emamian, E.S., Patterson, P.H., Shi, L., , Sidwell R (2002): Prenatal viral infection leads to pyramidal cell atrophy and macrocephaly in adulthood: implications for genesis of autism and schizophrenia. *Cellular and Molecular Neurobiology* 22:25-33.

Fatemi SH, Emamian, E.S., Kist, D., Sidwell, R.W., Nakajima, K., Akhter, P., Shier, A.,, Sheikh S, Bailey, K. (1999): Defective corticogenesis and reduction in Reelin immunoreactivity in cortex and hippocampus of prenatally infected neonatal mice. *Molecular Psychiatry* 4:145-154.

Fatemi SH, Pearce, D.A., Brooks, A.I., Sidwell, R.W. (2005): Prenatal viral infection in mouse causes differential expression of genes in brains of mouse progeny: a potential animal model for schizophrenia and autism. *Synapse* 57:91-99.

Fortier ME, Joober, R., Luheshi, G.N., Boksa, P. (2004): Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. *J Psychiatr Res* 38:335-345.

Fritz RS, Hayden FG, Calfee DP, Cass LM, Peng AW, Alvord WG, Strober W, Straus SE (1999): Nasal cytokine and chemokine responses in experimental influenza A virus infection: results of a placebo-controlled trial of intravenous zanamivir treatment. *Journal of Infectious Diseases* 180:586-593.

Hagberg H, Peebles D, Mallard C. (2002): Models of white matter injury: comparison of infectious, hypoxic-ischemic, and excitotoxic insults. *Mental Retardation and Developmental Disability Research Reviews* 8:30-38.

Hatten ME (1999): Central nervous system neuronal migration. *Annual Review in Neuroscience* 22:511-539.

Hatten ME, Alder, J., Zimmerman, K., Heintz, N. (1997): Genes involved in cerebellar cell specification and differentiation

. *Current Opinion in Neurobiology* 7:40-47.

Hatten ME, Heintz, N. (1995): Mechanisms of neural patterning and specification in the developing cerebellum

. *Annual Review of Neuroscience* 18:385-408.

Ho BC, Mola, C., Andreasen, N.C. (2004): Cerebellar dysfunction in neuroleptic naive schizophrenia patients: clinical, cognitive, and neuroanatomic correlates of cerebellar neurologic signs. *Biological Psychiatry* 55:1146-1153.

Hyman SL, Arndt, T.L., Rodier, P.M. (2006): Environmental agents and autism: Once and future associations. *International Review of Research in Mental Retardation* 30:171-194.

Kates WR, Burnette, C.P., Eliez, S., Strunge, L.A., Kaplan, D., Landa, R., Reiss, A.L., Pearlson, G.D. . (2004): Neuroanatomic variation in monozygotic twin pairs

discordant for the narrow phenotype for autism. *American Journal of Psychiatry* 161:539-546.

Kaufmann WE, Cooper, K.L., Mostofsky, S.H., Capone, G.T., Kates, W.R., Newschaffer, C.J., Bukelis, I., Stump, M.H., Jann, A.E., Lanham, D.C. (2003): Specificity of cerebellar vermian abnormalities in autism: a quantitative magnetic resonance imaging study. *Journal of Child Neurology* 18:463-470.

Kern JK (2003): Purkinje cell vulnerability and autism: a possible etiological connection. *Brain Development* 6:377-382.

Marenco S, Weinberger, D.R., Schreurs, B.G. (2003): Single-cue delay and trace classical conditioning in schizophrenia. *Biological Psychiatry* 53:390-402.

Mednick SA, Machon, R.A., Huttunen, M.O., Bonett, D. (1988): Adult schizophrenia following prenatal exposure to an influenza epidemic. *Archives of General Psychiatry* 45:189-192.

Merin N, Young, G.S., Ozonoff, S., Rogers, S.J. (2007): Visual Fixation Patterns during Reciprocal Social Interaction Distinguish a Subgroup of 6-Month-Old Infants At-Risk for Autism from Comparison Infants

. *Journal of Autism and Developmental Desorders* 37:108-121.

Meyer U, Feldon J, Schedlowski M, BK Y (2005): Towards an immuno-precipitated neurodevelopmental animal model of schizophrenia. *Neurosci Biobehav Rev* 29:913-947.

Meyer U, Feldon, J., Schedlowski, M., Yee, B.K. (2006): Immunological stress at the maternal-fetal interface: a link between neurodevelopment and adult psychopathology. *Brain, Behavior and Immunity* 20:378-388.

Miale IL, Sidman R.L. (1961): An autoradiographic analysis of histogenesis in the mouse cerebellum. *Experimental Neurology* 4:277-296.

Nowinski CV, Minshew, N.J., Luna, B., Takarae, Y., Sweeney, J.A. (2005): Oculomotor studies of cerebellar function in autism. *Psychiatry Research* 137:11-19.

Ozawa K, Hashimoto, K , Kishimoto T, Shimizu E, Ishikura H, M. I (2006): Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. *Biol Psychiatry* 59:546-554.

Ozol K, Hayden JM, Oberdick J, Hawkes R (1999): Transverse zones in the vermis of the mouse cerebellum. *J Comp Neurol* 412:95-111.

Palmen SJ, van Engeland, H., Hof, P.R., Schmitz, C. (2004): Neuropathological findings in autism. *Brain* 127:2572-2583.

Patterson PH (2002): Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. *Current Opinion in Neurobiology* 12:115-118.

Pierce K, Courchesne, E (2001): Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. *Biological Psychiatry* 49:655-664.

Pierson R, Alicata, D., Nopoulos, P., O'Leary, D., Andreasen, N.C. (2003): Cerebellar lobe morphology and its relation to symptoms in schizophrenia. *Schizophrenia Research* 60:205.

Ramnani N, Miall, C. (2001): Expanding cerebellar horizons. *Trends in Cognitive Science* 5:135-136.

Ritvo ER, Freeman, B.J., Scheibel, A.B., Duong, T., Robinson, H., Guthrie, D., Ritvo, A. (1986): Lower Purkinje cell counts in the cerebella of four autistic subjected: Initial findings of the UCLA-NSAC autopsy research center. *American Journal of Psychiatry* 143:7.

Rogers JH, Ciossek, T., Menzel, P., Pasquale, E.B. (1999): Eph receptors and ephrins demarcate cerebellar lobules before and during their formation. *Mechanisms of Development* 87:119-128.

Sarna JR, Hawkes, R. (2003): Patterned Purkinje cell death in the cerebellum. *Progress in Neurobiology* 70:473-507.

Schmahmann JD (2001): The cerebrocerebellar system: Anatomic substrates of the cerebellar contribution to cognition and emotion. *International Review of Psychiatry* 13:247-260.

Schutter DJ, van Honk, J. (2005): The cerebellum on the rise in human emotion. *Cerebellum* 4:290-294.

Sears LL, Andreasen, N.C., O'Leary, D.S. (2000): Cerebellar functional abnormalities in schizophrenia are suggested by classical eye blink conditioning. *Biological Psychiatry* 48:204-209.

Sears LL, Finn, P.R., Steinmetz, J.E. (1994): Abnormal classical eye-blink conditioning in autism. *Journal of Autism and Developmental Disorders* 24.

Shi L, Fatemi S.H., Sidwell, R.W., Patterson, P.H. (2003): Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. *Journal of Neuroscience* 23:297-302.

Smith SEP, Li, J., Garbett, K., Mirnics, K., Patterson, P.H. (2007): Maternal immune activation alters fetal brain development through interleukin-6. *Journal of Neuroscience* 27:10695-10702.

Steinmetz JE, Tracy, J.A., Green, J.T. (2001): Classical eyeblink conditioning: clinical models and applications. . *Integrative Physiological and Behavioral Science* 36:220-238.

Swiergiel AH, Dunn, A.J. (1999): The roles of IL-1, IL-6, and TNFalpha in the feeding responses to endotoxin and influenza virus infection in mice. *Brain, Behavior and Immunity* 13:252-265.

Swiergiel AH, Smagin, G.N., Dunn, A.J. (1997a): Influenza virus infection of mice induces anorexia: comparison with endotoxin and interleukin-1 and the effects of indomethacin. *Pharmacology, Biochemistry and Behavior* 57:389-396.

Swiergiel AH, Smagin, G.N., Johnson, L.J., Dunn, A.J. (1997b): The role of cytokines in the behavioral responses to endotoxin and influenza virus infection in mice: effects of acute and chronic administration of the interleukin-1-receptor antagonist (IL-1ra).

. *Brain Research* 776:96-104.

Takarae Y, Minshew, N.J., Luna, B., Sweeney, J.A. (2004): Oculomotor abnormalities parallel cerebellar histopathology in autism. *Journal of Neurology, Neurosurgery and Psychiatry* 75:1359-1361.

Tolbert DL, Clark, B.R. (2000): Olivocerebellar projections modify hereditary Purkinje cell degeneration. *Neuroscience* 101.

Uzman LL (1960): The histogenesis of the mouse cerebellum as studied by its tritiated thymidine uptake. *Journal of Comparative Neurology* 114:137-159.

Yuasa S, Kawamura, K., Ono, K., Yamakuni, T., Takahashi, Y. (1991): Development and migration of Purkinje cells in the mouse cerebellar primordium. *Anatomy and Embryology (Berl)* 184:195-212.

Zuckerman L, Rehavi, M., Nachman, R., Weiner, I. (2003): Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. *Neuropsychopharmacology* 28:1778-1789.

Zuckerman L, Weiner, I. (2005): Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. *Journal of Psychiatric Research* 39:311-323.

Chapter 5

Maternal Immune Activation Causes Abnormalities in Classical Eyeblink Conditioning in the Adult Offspring

Ka-Hung Lee[1], Stephen Smith[2], Soyun Kim[1], Paul H. Patterson[1] and Richard Thompson[2]

[1]Department of Biology, University of Southern California, Los Angeles, CA

[2]Division of Biology, California Institute of Technology, Pasadena, CA

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The work discussed in this chapter is ongoing; data collection will continue into the summer of 2008, and publication of the results will likely follow.

Abstract

Maternal immune activation (MIA) is a risk factor for both schizophrenia and autism, and a mouse model of maternal immune activation produces several deficits in the adult offspring that are reminiscent of the human diseases. The offspring of mice subjected to a single injection of the dsRNA poly(I:C) (MIA offspring) show a striking pathology in the cerebellum, the selective loss of Purkinje cells in lobules VI and VII, which is also often seen in autistic brains. Eyeblink conditioning is a cerebellum-dependent behavior that is abnormal in both schizophrenia and autism. We tested MIA offspring in a classical delay eyeblink conditioning paradigm at four and eight weeks of age. The MIA offspring show slightly impaired conditioning in the early phase of the test, and a slightly longer latency to produce a conditioned response (CR) after conditioned stimulus (CS) presentation. Most strikingly, the MIA offspring do not stop responding to the CS after repeated exposures to CS-alone trials, indicating a lack of reversal learning or extinction. The lack of extinction in these animals suggests hippocampal abnormalities, which have been reported in the MIA offspring. These results, taken together with a review of the literature, suggest that a closer examination of reversal learning in schizophrenia is warranted.

Introduction

Maternal infection is a well-established risk factor for both schizophrenia and autism in the offspring (Patterson, 2002; Fatemi, 2005; Brown, 2006; Meyer et al., 2006). The most convincing evidence for this comes from prospective studies of schizophrenia risk, which documented significantly increased incidence of schizophrenia in the

offspring of mothers who had experienced a respiratory infection or who had elevated levels markers of infection in their blood (Brown and Susser, 2002; Brown et al., 2004a; Brown et al., 2004b; Brown et al., 2005a; Babulas et al., 2006; Brown, 2006). The strongest case for maternal infection risk for autism comes from studies of rubella infection, which increased the risk >200-fold in the offspring (Desmond et al., 1967; Chess, 1977). Several smaller studies have identified autism cases following other maternal infections (Ciaranello and Ciaranello, 1995; Libbey et al., 2005). In rodent studies, MIA produces several behavioral changes in the adult offspring that are relevant for schizophrenia and autism (Shi et al., 2003; Zuckerman et al., 2003; Bakos et al., 2004; Fortier et al., 2004; Golan et al., 2005; Zuckerman and Weiner, 2005; Hava et al., 2006; Nyffeler et al., 2006; Ozawa et al., 2006; Smith et al., 2007). Because of its high construct and face validity, MIA is a useful model of human mental illness (Patterson, 2002, 2005).

While autism and schizophrenia are diagnosed by interviews as defined in the DSMIV, there are several secondary phenotypes that, while not diagnostic, are common in these disorders. For example, pre-pulse inhibition (PPI) is disrupted in schizophrenia (Wynn et al., 2004; Turetsky et al., 2007) and autism (Perry et al., 2006), as well as in people suffering from several other mental disorders. PPI is also disrupted in several animal models of schizophrenia, including the MIA model (Borrell et al., 2002; Shi et al., 2003; Ozawa et al., 2006; Romero et al., 2007). These behavioral markers are useful not only for validating animal models, bit also for understanding the neural mechanisms of the diseases.

Eyeblink conditioning is another behavior that is altered in both schizophrenia (Sears et al., 2000; Marenco et al., 2003; Brown et al., 2005b) and autism (Sears et al., 1994). Classical eyeblink conditioning involves pairing a conditioned stimulus (CS), usually an audible tone, with an aversive, unconditioned stimulus (US), either an air puff to the eye or an electric shock to the orbital muscle. The neural circuitry that mediates eyeblink conditioning is well-characterized and is conserved across mammalian species (Woodruff-Pak and Steinmetz, 2000). Simple delay conditioning, in which the US immediately follows the CS, depends upon a cerebellar circuit in which the interpositus nucleus integrates information about the US, coming in via the inferior olive and the climbing fibers, and the CS, coming from the pontine nucleus and mossy fibers (De Zeeuw and Yeo, 2005; Wada et al., 2007). More complex tasks such as reversal learning (extinction) or trace conditioning involve the hippocampus and pre-frontal cortex, as well as cerebellar circuitry (Robleto et al., 2004; Robleto and Thompson, 2008).

In a single published study of delay eyeblink conditioning in autism, the acquisition of the conditioned response (CR) was faster than in age-matched controls, and the onset and peak latency of the conditioned response was faster (Sears et al., 2000). This suggests that autistic children are *better* at this particular type of learning, although the decreased peak latency of the CR may be maladaptive, since the peak eyeblink response occurred before the arrival of the airpuff. Furthermore, the age-related performance improvement that was observed in controls was not noted in subjects with autism, suggesting a possible early maturation of this response, which correlates the early brain overgrowth, followed by normalization of brain volume, that has been described in autism (Courchesne, 2004; Courchesne et al., 2007). In three studies of eyeblink

conditioning in schizophrenia, mixed results have been reported (see Table 1). Faster (Sears et al., 2000), normal (Marenco et al., 2003), or slower (Brown et al., 2005b) rates of CR acquisition, as well as longer (Marenco et al., 2003) and shorter (Sears et al., 2000; Brown et al., 2005b) CR peak latency have been reported. These inconsistent results could be related to the medication state of the subjects.

In a mouse model of MIA, the offspring have cerebellar abnormalities, specifically a loss of Purkinje cells in lobule VII of the cerebellum (Shi et al., submitted). This corresponds with data showing a spatially selective loss of Purkinje cells in post-mortem autistic brains (Pierce and Courchesne, 2001; Akshoomoff et al., 2004; Allen et al., 2004; Kates et al., 2004; Palmen et al., 2004), as well as abnormal cerebellar MRI data in both schizophrenia (Ho et al., 2004; Bottmer et al., 2005; Picard et al., 2008) and autism (Pierce and Courchesne, 2001; Akshoomoff et al., 2004; Allen et al., 2004; Kates et al., 2004). However, some studies report negative findings in this regard (reviewed by Palmen et al., 2004). Since the MIA model replicates this key pathology, and the cerebellum is critical for eyeblink conditioning, and since affected humans display eyeblink conditioning abnormalities, we characterized classical eyeblink conditioning in the mouse MIA model.

Methods

Production of animals Adult C57BL/6J mice were housed in the Univeristy of Southern California animal facility on a 12/12 hour light cycle. Females were placed with males overnight, and the presence of a vaginal plug marked E0. On E12.5, pregnant females were injected with 20 mg/kg of poly(I:C) (5 ml/kg; potassium salt of polyIC; Sigma)

intraperitoneally, or with the equivalent volume of sterile saline. Sickness behavior was confirmed in poly(I:C)-treated females; otherwise they were excluded from the experiment. Females gave birth at the usual time and the offspring were weaned at three weeks. In two separate experiments, animals were subjected to the following eyeblink conditioning criteria at either 8 (N = 8 control, 14 poly(I:C)) or 4 weeks of age (N = 7 control, 6 poly(I:C)).

Surgery. Under anesthesia (ketamine, 80 mg/kg; xylazine, 20 mg/kg; subcutaneous), 4 Teflon-coated, stainless steel electordes were implanted at the upper left obicularis oculi muscle (2 for periorbital shock; 2 for electromyograph). A 4-pin connector was soldered to the 4 electrodes. Dental acrylic was applied hold the whole assembly together as a headstage and attach the assembly to the skull.

Eyeblink conditioning. One week after surgery, the adult offspring were habituated (H) to the chamber for 1 day without tone or shock, and spontaneous eyeblink activity recorded. The mice were then trained with 250 ms delay eyeblink conditioning for 5 days (A1-A5, the acquisition period) with a tone (1 kHz, 85 dB) as the CS, and co-terminating with a mild, 100 ms shock (100 Hz, biphasic) as the US. Each day of training consisted of 100 trials (80% CS-US paired). One hundred CS-alone trials were then given daily for the following 10 days (E1-E10, the extinction period).

Sensory tests. Auditory brain-stem responses (ABR) were recorded to test for auditory sensitivity. While the mice were under anesthesia (see Surgery), two platinum electrodes were implanted subcutaneously on the vertex of the skull and ventrolateral to one of the ear pinnae. The grounding electrode was implanted in the tail. Each mouse was tested

with 2,000 tone-on (85 dB, 1kHz, 10 ms) trials and followed by another 2,000 tone-off (0 dB) trials as the control. A tail-flick test was performed to test for nociception. Each mouse was put into a plastic restrainer with the tail resting on a hot plate (52 °C). The onset latency of tail flick was timed with a stopwatch. The test was performed on each mouse 3 times at 1 min intervals.

Data Analysis. Conditioned response analysis was preformed as previously described (Chen et al., 1996).

Statistical Analysis. Comparisons between two groups, or between two time-points of a single group, were made using the Student t-test. Comparisons between groups involving multiple time-points were made using two-way ANOVA, with prenatal treatment and time the main variables.

Results

Acquisition of the CR. The acquisition of the CR was measured using the percent of CS trails in which the animals showed a CR (%CR) and the amplitude of the CR, as measured by the implanted electrodes. In adult animals (8 weeks old), both of these measures yield no significant differences between the control and poly(I:C) groups (Fig. 1A,B). If statistical analysis is limited to early training (days A1-A4), before the learning plateau is reached, the poly(I:C) offspring show a significant deficit ($P = 0.048$). In humans, correlated with the early-maturation phenotype displayed by autistic children, comparisons of younger individuals yield differences that are larger in magnitude (Sears et al., 1994). Therefore, we tested another group of animals at the earliest possible time point (4 weeks of age) that was technically possible with this assay. These animals also show no significant differences between the control and poly(I:C) treated offspring

(figure 1C,D). However, as with the adult animals, if the analysis is limited early acquisition (days 1-4), the poly(I:C) group shows a trend towards a significant deficit ($P = 0.07$).

Extinction of the CR. The most striking difference between the experimental groups is the lack of reversal learning, or extinction of the CR in the poly(I:C) offspring. As measured by both %CR and by CR amplitude after ten days of reversal training, control animals show a markedly diminished CR (Fig. 2). In contrast, poly(I:C) offspring show a lack of reversal learning, with no significant decline in %CR or CR amplitude after ten days of extinction training. A similar deficit is observed in juvenile animals.

Timing of the CR. The adult offspring of poly(I:C)-treated mothers display a significant shift towards a later onset of the CR ($p < 0.011$), as well as a later peak CR ($p < 0.003$) (Fig. 3). This effect is only seen during the acquisition phase, and is not present during the extinction phase. In the juvenile animals, the sample size ($N = 6$) was perhaps too small to observe this effect.

Sensory parameters. The differences in conditioning are not apparently due to sensory problems in the poly(I:C) offspring, as there are no detectable differences in sensory parameters between control and poly(I:C) mice (Fig. 4). The amplitude of the UR to the orbital shock (the US, Fig. 4A) and the level of US necessary to induce an eyeblink response (Fig. 4B) are similar between the groups, as is the latency to tailflick during the hotplate test (Fig. 4D), indicating a similar pain threshold between the two groups.

Finally, the auditory brainstem response is similar in the two groups, indicating normal hearing (Fig. 4D).

Discussion

While the offspring of poly(I:C)-treated mice exhibit normal sensory parameters, they display slightly impaired eyeblink conditioning, and slightly increased latency to produce a CR after CS presentation. This could reflect subtle abnormalities in the cerebellar circuitry, as suggested by the Purkinje cell deficit in these animals (Shi et al., submitted). More striking is the lack of extinction learning in the MIA offspring. After ten days of extinction trials, when control mice show a significant decline in the response to the CS, MIA offspring continue to respond strongly to the CS.

There are several possible brain regions that could be responsible for the extinction deficit in the MIA offspring. Extinction is thought to reflect the active learning of a "CS-irrelevant" association, rather than a forgetting of the trained "CS-relevant" association. Mice that have undergone conditioning followed by extinction subsequently re-condition significantly faster than naïve animals (for review see (Falls, 1998). Interestingly, MIA offspring also show impaired learning of a "CS-irrelevant" association in the latent inhibition (LI) paradigm (Zuckerman et al., 2003; Smith et al., 2007). In that case, control mice that are pre-exposed to a tone display diminished fear-conditioning when that tone is subsequently paired with a footshock, while poly(I:C) offspring condition to the tone regardless of prior exposure. Several lesion and pharmacological studies demonstrate that a functioning hippocampus and normal dopaminergic function are critical for LI (reviewed by (Weiner, 2003)). Moreover, an intact hippocampus is

critical for extinction of the CR in eyeblink conditioning (reviewed by (Robleto et al., 2004). Most notably, hippocampal-lesioned rabbits can learn the CS-US association (reflecting the sufficiency of the cerebellum for acquisition), but do not show any signs of extinction in subsequent CS-alone trails (Akase et al., 1989).

The NMDA receptor is also critical for extinction learning in the eyeblink paradigm, although dopaminergic signaling is not (Scavio et al., 1992). Trained animals given the NMDA antagonist ketamine show accelerated acquisition and decreased extinction of the CR (Scavio et al., 1992). Furthermore, trained animals given NMDA antagonists MK-801 or phencyclidine (PCP) during extinction training show a complete lack of extinction, similar to that seen in our MIA offspring (Thompson and Disterhoft, 1997). In contrast, post-training injections of amphetamine, chloropromazine or scopolamine, which impair conditioning, have no effect on extinction (Scavio et al., 1992), suggesting that dopaminergic signaling is not involved. These pharmacological agents have only been administered systemically in the extinction experiments, not locally as with some acquisition experiments, so it is not possible to localize these effects to specific brain structures (Robleto et al., 2004). Intriguingly, NMDA receptor abnormalities have been reported in the hippocampus of offspring of rats given IL-6 injections during pregnancy (Samuelsson et al., 2006), and IL-6 is implicated in mediating the effects of poly(I:C) MIA (Smith et al., 2007). In addition, hippocampal signaling can modulate dopamine release in a chemically-induced schizophrenia model (Lodge and Grace, 2007), which implies that a fundamental deficit in the hippocampus, perhaps reflected in eyeblink extinction impairments, could also impair dopamine-dependent behaviors, such as LI.

The Purkinje cell deficit observed in MIA offspring (Shi et al., submitted) is also a plausible cellular basis of the eyeblink conditioning abnormalities. MIA offspring have a 20 to 30 percent reduction in the number of Purkinje cells in lobule VII of the cerebellar cortex. However, in the eyeblink paradigm, Purkinje-cell deficient mice, in which all Purkinje cells in the cerebellar cortex die when the animal is two weeks old, are still capable of (slow) acquisition learning, and, importantly, normal extinction (Chen et al., 1999). This suggests that the main extinction deficit seen in MIA offspring is not due to the relatively subtle cerebellar pathology, although the slightly impaired learning observed in our mice could be related to this deficit.

The relationship of the eyeblink abnormalities that we observe in MIA offspring to findings in schizophrenia and autism is not completely clear at present. The published study of eyeblink conditioning in autism suggests a different phenotype than we observe, with faster acquisition and a shorter latency of the CR (Sears et al., 1994). A more recent study on individuals with fragile X syndrome suggests impairments in acquisition of the CR as well as longer CS-response latency, consistent with our data (Tobia and Woodruff-Pak, 2007). Fragile X is relevant because 15-25% percent of affected individuals also display autistic-like traits (Bailey et al., 2001). The schizophrenia eyeblink literature is mixed, with inconsistent results reported among different groups (Table 1). Perhaps the most reliable study is by Sears et al. (2000) since their subjects had been medication-free for at least three weeks at the time of testing, and anti-psychotic drugs are known to effect conditioning. In fact, Marenco et al (2003) report their three un-medicated subjects showed a lower percent CR than their seven medicated patients (66% vs 82%), indicating that medication state of the subjects cannot be ignored. Sears et al. (2000) reported

results similar to those in the autism study, which incidentally shared the same first author, reporting faster acquisition and shorter CR latency. The Brown et al. study (2005b) used all medicated patients and reported impaired acquisition and shorter CR latency. The Marenco study (2003) used a mix of medicated and medication-free patients, and reported no difference in acquisition and a longer CR latency. It should be noted that (Stevens et al., 2002) also briefly mentioned measuring normal eyeblink conditioning in both medicated and unmedicated schizophrenics, but data was not shown and conditioning parameters were not discussed.

Critical to the present discussion is the extinction data that, in all three schizophrenia studies, was reported as normal. However, examination of the only study to actually present their extinction data reveals an apparent lack of extinction in schizophrenic subjects (see Fig. 5 from Brown et al., 2005b). Interpretation of the results are confounded by the lower acquisition rate of schizophrenics in this experiment, but the differences between pre- and post-extinction CR rate in the schizophrenic group do not appear to be significant. While our mouse data does not fit neatly with any single study population, the apparent lack of extinction in schizophrenic individuals deserves further investigation.

Table 1: Published reports of eyeblink conditioning abnormalities in schizophrenia, autism and fragile X. As of 2008, only one published report exists for autism and one for fragile X. Note the variability among the schizophrenia data, possibly due to a difference in anti-psychotic medication status among the subjects. Yrs years of age; N.R. not reported; sig. significantly; S.D. standard deviation of the age, rng range of the age.

Citation	Patient Population	Acquisition: % CR	Acquisition: CR amplitude	CR Latency	Extinction
Sears et al, 1994	11 autistic children 12yrs (rng 7-22)	Higher in early trials, same at end of training	Reached asymptotic values faster	Shorter	More rapid in early trials, then normal
Tobia and Woodruff-Pak, 2007	20 Fragile X patients, 45yrs (rng 17-77)	Lower in all trials	N.R.	Longer	N.R.
Sears et al, 2000	15 medication-free (3wks) schizophrenics 32yrs (rng 20-49)	Higher in all trials, faster acquisition	N.R.	Shorter, but not sig. different when corrected for %CR differences	Reported normal, data not shown.
Marenco et al, 2003	10 schizophrenics, 3 of which were medication-free, 41 yrs (S.D.9.7)	No difference	N.R.	Longer	Reported normal, data not shown
Brown et al, 2005	13 schizophrenics, all medicated, 42yrs (S.D. 9.5)	Lower in all trials	No significant differences	Shorter	Reported as normal, but data shown in figures suggests a lack of extinction in patients

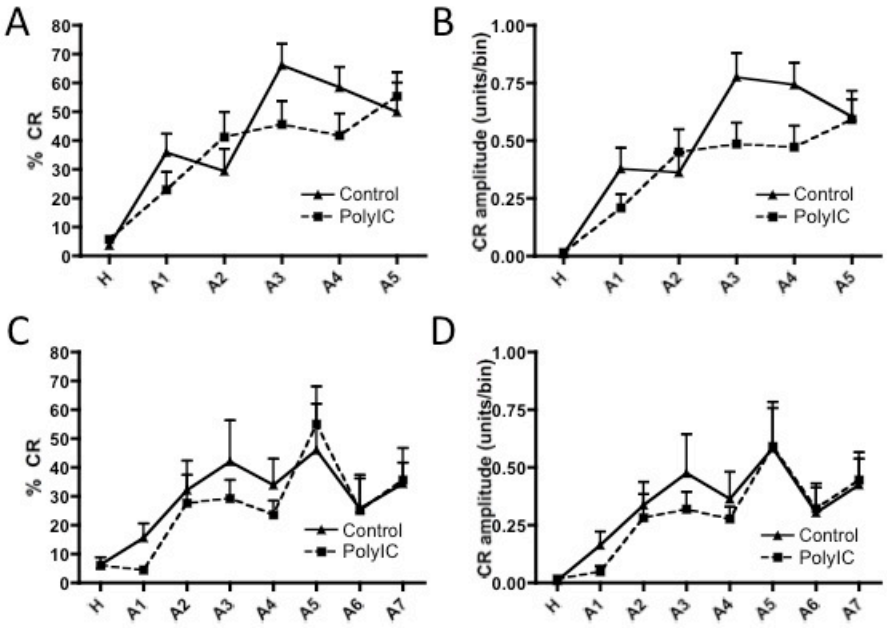


Figure 1. Acquisition of the conditioned eyeblink response is altered in MIA offspring. The percent of tone-only trails in which animals responded with a CR (A,C), and the amplitude of the CR (B,D) are shown for both adult (8 weeks old; A,B) and juvenile (4 weeks old; C,D) animals. Overall, there are no significant differences for either measure of acquisition in either age group. However, if analysis is limited to the early training period (days A1-A4), there is a significant difference between MIA and control adult offspring in both %CR and CR amplitude ($P < 0.05$) and a trend towards significance in the juveniles ($P < 0.07$ and 0.17, respectively). Overall, these data indicate a slight impairment in eyeblink conditioning in the MIA offspring.

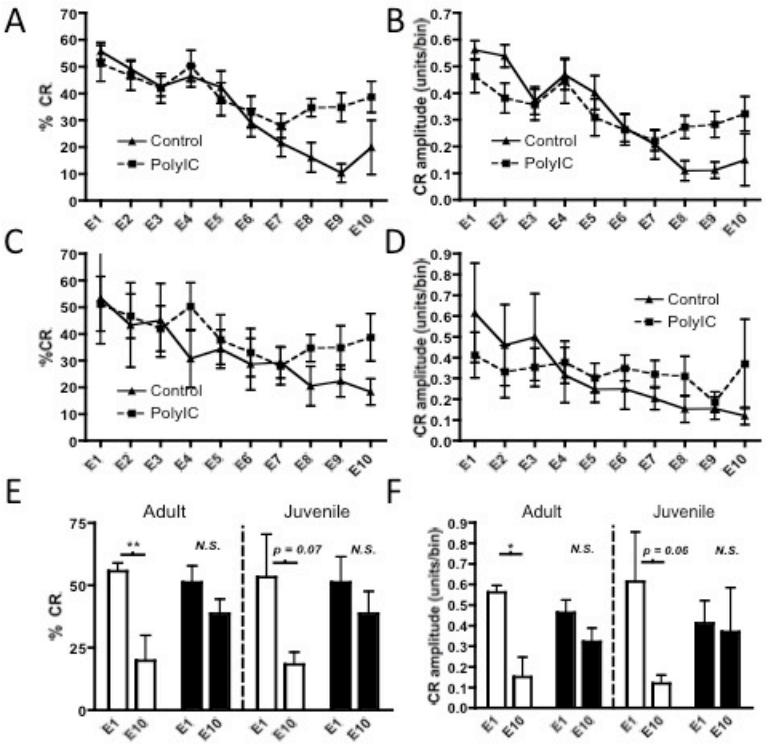


Figure 2. Extinction of the conditioned eyeblink response is altered in MIA offspring. The percent of tone-only trails to which animals respond with a CR (A,C) and the amplitude of the CR (B,D) are shown for both adult (A,B) and juvenile (C,D) animals. The MIA offspring show a significant lack of extinction behavior, as measured by both %CR and CR amplitude in the adults ($P < 0.05$). There is also a significant lack of extinction measured by CR amplitude in the juveniles ($P < 0.024$). Furthermore, when the first (E1) and last (E10) days of extinction trials are compared, control adult animals show significant differences ($p < 0.05$) in both %CR (E) and CR amplitude (F), while the adult and juvenile MIA offspring do not.

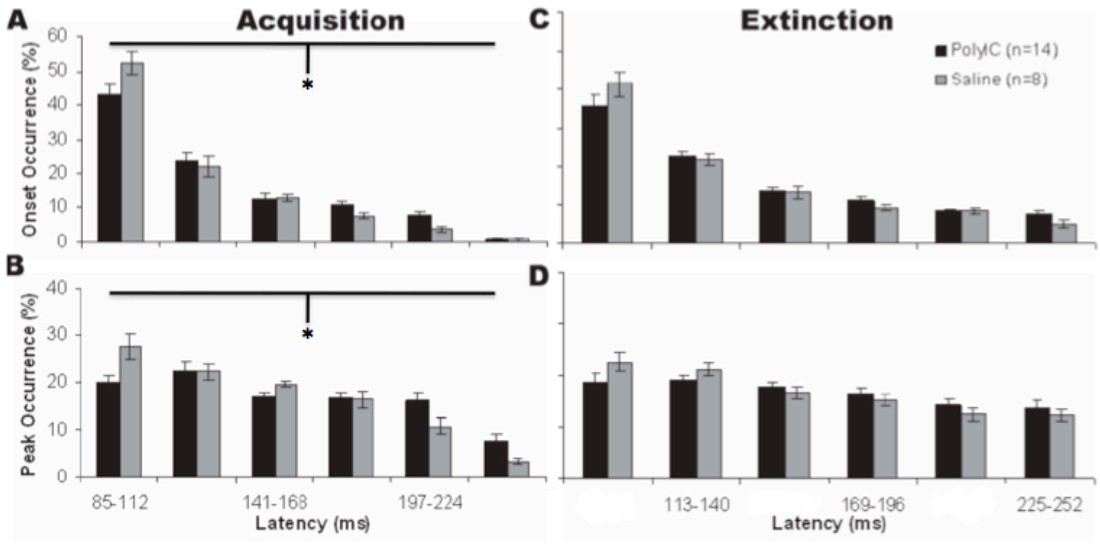


Figure 3. Timing of the CR is altered in MIA offspring. The latency between the tone and the onset (A,C) or peak of the CR (B,D) is significantly longer in MIA offspring during acquisition (A,B) but not during extinction (C,D). The latency values are binned, and the bars represent the percent of CRs that fall into each timing bin. * Two-way ANOVA reaveals a significant effect of both time and treatment in A and B ($p < 0.05$).

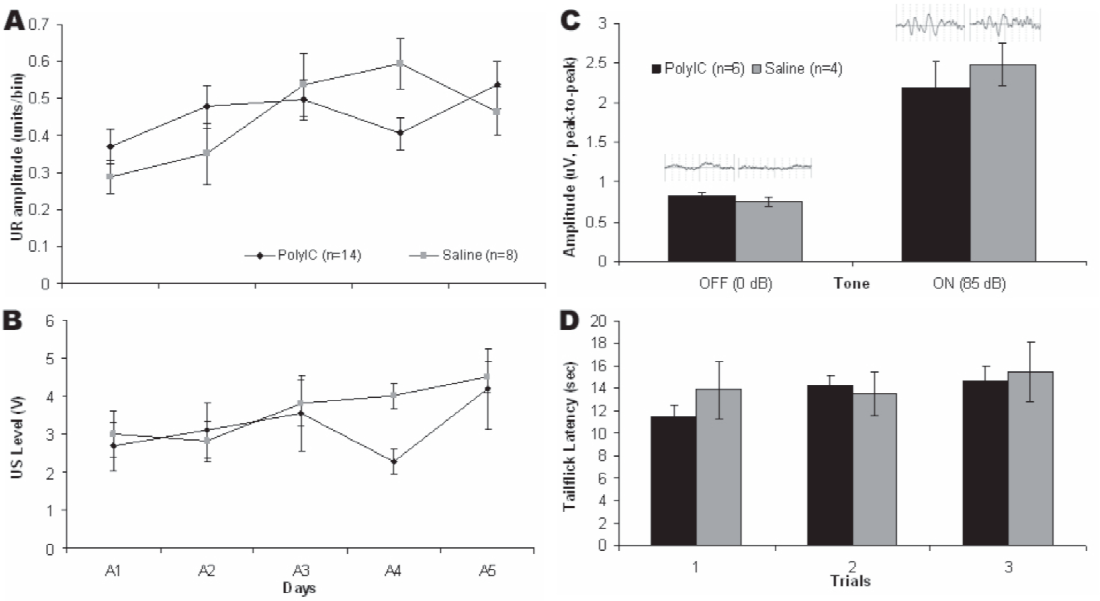


Figure 4. Sensory parameters are normal in MIA offspring. (A) The amplitude of the eyeblink (UR) in response to the US-alone trials is similar in both MIA and control offspring. (B) When the amplitude of the orbital shock, the US, is adjusted daily to achieve the desired behavioral response (an eyeblink) the US required is similar for both groups. (C) The auditory brain stem response in response to both no-stimulus (left) and an 85 db tone (right) is similar in both groups. Representative brain stem response traces are shown above the bars. (D) The latency to flick the tail in the hot-plate nociception test is similar in both groups.

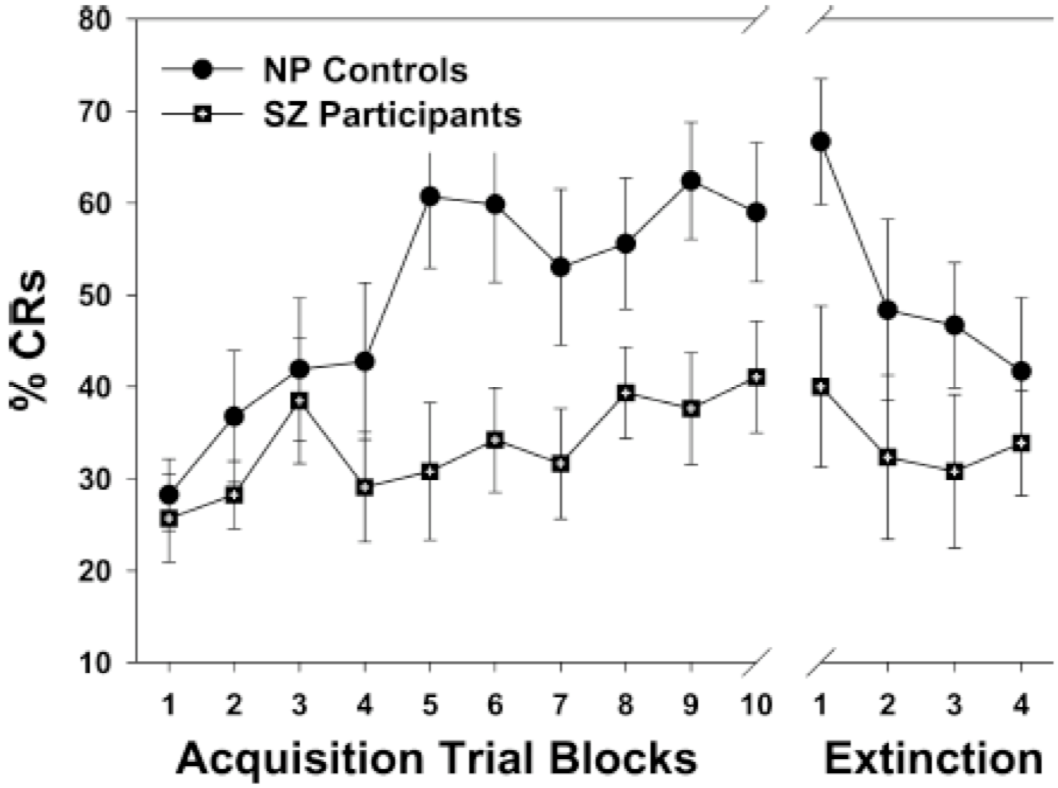


Figure 5. The unmodified Figure 2 from the study by Brown et al. (2005b) is shown. While the authors conclude that both schizophrenics (SZ) and non-psychiatric (NP) controls display normal extinction, comparison of the extinction trials one and four suggests that extinction may be impaired in the schizophrenic group. However, the analysis is complicated by the lower peak rate of CRs in schizophrenics vs. controls.

References

Akase E, Alkon DL, Disterhoft JF (1989) Hippocampal lesions impair memory of short-delay conditioned eye blink in rabbits. Behav Neurosci 103:935-943.

Akshoomoff N, Lord C, Lincoln AJ, Courchesne RY, Carper RA, Townsend J, Courchesne E (2004) Outcome classification of preschool children with autism spectrum disorders using MRI brain measures. J Am Acad Child Adolesc Psychiatry 43:349-357.

Allen G, Muller RA, Courchesne E (2004) Cerebellar function in autism: functional magnetic resonance image activation during a simple motor task. Biol Psychiatry 56:269-278.

Babulas V, Factor-Litvak P, Goetz R, Schaefer CA, Brown AS (2006) Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. Am J Psychiatry 163:927-929.

Bailey DB, Jr., Hatton DD, Skinner M, Mesibov G (2001) Autistic behavior, FMR1 protein, and developmental trajectories in young males with fragile X syndrome. J Autism Dev Disord 31:165-174.

Bakos J, Duncko R, Makatsori A, Pirnik Z, Kiss A, Jezova D (2004) Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. Ann N Y Acad Sci 1018:281-287.

Borrell J, Vela JM, Arevalo-Martin A, Molina-Holgado E, Guaza C (2002) Prenatal immune challenge disrupts sensorimotor gating in adult rats. Implications for the etiopathogenesis of schizophrenia. Neuropsychopharmacology 26:204-215.

Bottmer C, Bachmann S, Pantel J, Essig M, Amann M, Schad LR, Magnotta V, Schroder J (2005) Reduced cerebellar volume and neurological soft signs in first-episode schizophrenia. Psychiatry Res 140:239-250.

Brown AS (2006) Prenatal infection as a risk factor for schizophrenia. Schizophr Bull 32:200-202.

Brown AS, Susser ES (2002) In utero infection and adult schizophrenia. Ment Retard Dev Disabil Res Rev 8:51-57.

Brown AS, Schaefer CA, Quesenberry CP, Jr., Liu L, Babulas VP, Susser ES (2005a) Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. Am J Psychiatry 162:767-773.

Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP, Susser ES (2004a) Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 61:774-780.

Brown AS, Hooton J, Schaefer CA, Zhang H, Petkova E, Babulas V, Perrin M, Gorman JM, Susser ES (2004b) Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry 161:889-895.

Brown SM, Kieffaber PD, Carroll CA, Vohs JL, Tracy JA, Shekhar A, O'Donnell BF, Steinmetz JE, Hetrick WP (2005b) Eyeblink conditioning deficits indicate timing and cerebellar abnormalities in schizophrenia. Brain Cogn 58:94-108.

Chen L, Bao S, Thompson RF (1999) Bilateral lesions of the interpositus nucleus completely prevent eyeblink conditioning in Purkinje cell-degeneration mutant mice. Behav Neurosci 113:204-210.

Chen L, Bao S, Lockard JM, Kim JK, Thompson RF (1996) Impaired classical eyeblink conditioning in cerebellar-lesioned and Purkinje cell degeneration (pcd) mutant mice. J Neurosci 16:2829-2838.

Chess S (1977) Follow-up report on autism in congenital rubella. J Autism Child Schizophr 7:69-81.

Ciaranello AL, Ciaranello RD (1995) The neurobiology of infantile autism. Annu Rev Neurosci 18:101-128.

Courchesne E (2004) Brain development in autism: early overgrowth followed by premature arrest of growth. Ment Retard Dev Disabil Res Rev 10:106-111.

Courchesne E, Pierce K, Schumann CM, Redcay E, Buckwalter JA, Kennedy DP, Morgan J (2007) Mapping early brain development in autism. Neuron 56:399-413.

De Zeeuw CI, Yeo CH (2005) Time and tide in cerebellar memory formation. Curr Opin Neurobiol 15:667-674.

Desmond MM, Wilson GS, Melnick JL, Singer DB, Zion TE, Rudolph AJ, Pineda RG, Ziai MH, Blattner RJ (1967) Congenital rubella encephalitis. Course and early sequelae. J Pediatr 71:311-331.

Falls WA (1998) Extinction: A review of theory and evidence suggesting that memories are not erased with non-reinforcement. in Learning and Behavior Therapy (ed W O'Donohue) pp 205-229 Allyn and Bacon, Boston, Ma.

Fatemi SH (2005) Prenatal human influenza viral infection, brain development and schizophrenia. in Neuropsychiatric disorders and infeciton (ed S H Fatemi) pp 66-83 Taylor and Francis, UK.

Fortier ME, Joober R, Luheshi GN, Boksa P (2004) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res 38:335-345.

Golan HM, Lev V, Hallak M, Sorokin Y, Huleihel M (2005) Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy. Neuropharmacology 48:903-917.

Hava G, Vered L, Yael M, Mordechai H, Mahoud H (2006) Alterations in behavior in adult offspring mice following maternal inflammation during pregnancy. Dev Psychobiol 48:162-168.

Ho BC, Mola C, Andreasen NC (2004) Cerebellar dysfunction in neuroleptic naive schizophrenia patients: clinical, cognitive, and neuroanatomic correlates of cerebellar neurologic signs. Biol Psychiatry 55:1146-1153.

Kates WR, Burnette CP, Eliez S, Strunge LA, Kaplan D, Landa R, Reiss AL, Pearlson GD (2004) Neuroanatomic variation in monozygotic twin pairs discordant for the narrow phenotype for autism. Am J Psychiatry 161:539-546.

Libbey JE, Sweeten TL, McMahon WM, Fujinami RS (2005) Autistic disorder and viral infections. J Neurovirol 11:1-10.

Lodge DJ, Grace AA (2007) Aberrant hippocampal activity underlies the dopamine dysregulation in an animal model of schizophrenia. J Neurosci 27:11424-11430.

Marenco S, Weinberger DR, Schreurs BG (2003) Single-cue delay and trace classical conditioning in schizophrenia. Biol Psychiatry 53:390-402.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Nyffeler M, Meyer U, Yee BK, Feldon J, Knuesel I (2006) Maternal immune activation during pregnancy increases limbic GABAA receptor immunoreactivity in the adult offspring: implications for schizophrenia. Neuroscience 143:51-62.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Palmen SJ, van Engeland H, Hof PR, Schmitz C (2004) Neuropathological findings in autism. Brain 127:2572-2583.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Patterson PH (2005) Maternal influenza infection leads to neuropathology and behavioral abnormalities in adult offspring. Neuropsychopharmacology 30:S9-S9.

Perry W, Minassian A, Lopez B, Maron L, Lincoln A (2006) Sensorimotor Gating Deficits in Adults with Autism. Biol Psychiatry.

Picard H, Amado I, Mouchet-Mages S, Olie JP, Krebs MO (2008) The role of the cerebellum in schizophrenia: an update of clinical, cognitive, and functional evidences. Schizophr Bull 34:155-172.

Pierce K, Courchesne E (2001) Evidence for a cerebellar role in reduced exploration and stereotyped behavior in autism. Biol Psychiatry 49:655-664.

Robleto K, Thompson RF (2008) Extinction of a classically conditioned response: red nucleus and interpositus. J Neurosci 28:2651-2658.

Robleto K, Poulos AM, Thompson RF (2004) Brain mechanisms of extinction of the classically conditioned eyeblink response. Learn Mem 11:517-524.

Romero E, Ali C, Molina-Holgado E, Castellano B, Guaza C, Borrell J (2007) Neurobehavioral and immunological consequences of prenatal immune activation in rats. Influence of antipsychotics. Neuropsychopharmacology 32:1791-1804.

Samuelsson AM, Jennische E, Hansson HA, Holmang A (2006) Prenatal exposure to interleukin-6 results in inflammatory neurodegeneration in hippocampus with NMDA/GABA(A) dysregulation and impaired spatial learning. Am J Physiol Regul Integr Comp Physiol 290:R1345-1356.

Scavio MJ, Clift PS, Wills JC (1992) Posttraining effects of amphetamine, chlorpromazine, ketamine, and scopolamine on the acquisition and extinction of the rabbit's conditioned nictitating membrane response. Behav Neurosci 106:900-908.

Sears LL, Finn PR, Steinmetz JE (1994) Abnormal classical eye-blink conditioning in autism. J Autism Dev Disord 24:737-751.

Sears LL, Andreasen NC, O'Leary DS (2000) Cerebellar functional abnormalities in schizophrenia are suggested by classical eyeblink conditioning. Biol Psychiatry 48:204-209.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 23:297-302.

Shi L, Smith SE, Malkova N, Tse D, Patterson PH (submitted) Activation of the maternal immune system alters cerebellar development in the offspring.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Stevens A, Schwarz J, Schwarz B, Ruf I, Kolter T, Czekalla J (2002) Implicit and explicit learning in schizophrenics treated with olanzapine and with classic neuroleptics. Psychopharmacology (Berl) 160:299-306.

Thompson LT, Disterhoft JF (1997) N-methyl-D-aspartate receptors in associative eyeblink conditioning: both MK-801 and phencyclidine produce task- and dose-dependent impairments. J Pharmacol Exp Ther 281:928-940.

Tobia MJ, Woodruff-Pak DS (2007) Acquisition, timing and long-term retention of conditioned eyeblink responding in subjects with fragile X syndrome. 2007 Society for Neuroscience poster presentation.

Turetsky BI, Calkins ME, Light GA, Olincy A, Radant AD, Swerdlow NR (2007) Neurophysiological Endophenotypes of Schizophrenia: The Viability of Selected Candidate Measures. Schizophr Bull 33:69-94.

Wada N, Kishimoto Y, Watanabe D, Kano M, Hirano T, Funabiki K, Nakanishi S (2007) Conditioned eyeblink learning is formed and stored without cerebellar granule cell transmission. Proc Natl Acad Sci U S A 104:16690-16695.

Weiner I (2003) The "two-headed" latent inhibition model of schizophrenia: modeling positive and negative symptoms and their treatment. Psychopharmacology (Berl) 169:257-297.

Woodruff-Pak DS, Steinmetz JE (2000) Eyeblink classical conditioning: Animal models. Kluwer Academic Publishers, Boston, MA.

Wynn JK, Dawson ME, Schell AM, McGee M, Salveson D, Green MF (2004) Prepulse facilitation and prepulse inhibition in schizophrenia patients and their unaffected siblings. Biol Psychiatry 55:518-523.

Zuckerman L, Weiner I (2005) Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. J Psychiatr Res 39:311-323.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Chapter 6

Maternal Immune Activation Alters Hippocampal-Dependent Behavior and Dopamine Responses in the Adult Offspring

Hiroshi Ito, Stephen Smith, Paul H. Patterson and Erin Schumann

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

The work discussed in this chapter is ongoing; data collection will continue into the summer of 2008, and publication of the results will likely follow.

Pharmacological evidence suggests that abnormal dopaminergic signaling plays an important role in the pathogenesis of schizophrenia. Using a mouse model of schizophrenia with high face and construct validity, the maternal immune activation model, we characterized dopaminergic signaling in the hippocampus using slice electrophysiology in the offspring of mice subjected to immune activation with the dsRNA, poly(I:C), during pregnancy. We also assayed several hippocampal-dependent behaviors. The adult offspring of poly(I:C)-treated females show normal long-term potentiation (LTP) at Schaffer collateral synapses. However, they show a reduced frequency and increased amplitude of miniature excitatory post-synaptic potentials (mEPSPs), suggesting pre-synaptic abnormalities. Most strikingly, the offspring of poly(I:C)-treated mothers show an increased sensitivity to the effects of exogenously-applied dopamine. Compared to control mice, they also demonstrate enhanced spatial learning in the Morris water maze, and enhanced novelty discrimination in an object switching task. We propose that the hypersensitivity to dopamine that we observe in the hippocampus causes a functional improvement in the dopamine-dependant hippocampal high-pass filter, leading to enhanced behavioral performance. However, this abnormal signaling could also be maladaptive, leading to some of the abnormal behaviors observed in both the maternal immune activation rodent model, and in schizophrenia.

Introduction

Maternal infection is a risk factor for both schizophrenia and autism in the offspring (Hyman et al., 2005; Brown, 2006). The most convincing evidence for this comes form a series of studies using archived maternal blood samples, or detailed examination of maternal medical records, which documented significantly increased

incidence of schizophrenia in the offspring of mothers with elevated levels of infection markers in their blood (Brown and Susser, 2002; Brown et al., 2004a; Brown et al., 2004b; Brown et al., 2005; Babulas et al., 2006). Similarly, prenatal viral infection may be an important non-genetic cause of autism (Ciaranello and Ciaranello, 1995). The most compelling example for autism comes from studies of maternal rubella infection, which increases the risk of autism in the offspring > 200-fold (Desmond et al., 1967; Chess, 1977) Several smaller studies have associated autism cases with other maternal infections (Ciaranello and Ciaranello, 1995; Libbey et al., 2005). In animal studies, maternal immune activation (MIA) produces several relevant behavioral changes in the adult offspring (Shi et al., 2003; Zuckerman et al., 2003; Bakos et al., 2004; Fortier et al., 2004b; Golan et al., 2005; Hava et al., 2006; Nyffeler et al., 2006; Ozawa et al., 2006; Smith et al., 2007). Because of its high construct and face validity, MIA in rodents is a useful model of human mental disease (Patterson, 2002, 2005).

In addition to the behavioral abnormalities, which are post-pubertal in onset and can be corrected by anti-psychotic drugs, the offspring of MIA rodents share several important pharmacological and histological features with schizophrenic patients, including abnormalities in dopaminergic function. Extensive pharmacological evidence points to dysfunction of the dopamine system as being critical in the pathogenesis of schizophrenia (Abi-Dargham, 2004; Meisenzahl et al., 2007). For example, the clinical efficacy of anti-psychotic drugs is well correlated with their affinity for the D_2 receptor (Seeman et al., 1976; Kapur and Mamo, 2003). Moreover, PET imaging shows a higher baseline occupancy of D_2 receptors in the striatum of schizophrenic individuals, suggesting abnormally high dopaminergic activity (Abi-Dargham et al., 2000; Kegeles et

al., 2008). In animal models of the maternal infection risk factor, the offspring of pregnant rodents whose immune systems were activated show several abnormalities in dopaminergic signaling and dopamine-dependent behaviors. For example, in the synthetic dsRNA poly(I:C) MIA model, the adult offspring display enhanced amphetamine-induced locomotion (Zuckerman et al., 2003; Fortier et al., 2004a; Ozawa et al., 2006), a lack of latent inhibition (Zuckerman et al., 2003; Smith et al., 2007), and abnormal levels of dopamine and dopamine metabolites in the brain (Ozawa et al., 2006).

The role of the hippocampus in modulating dopaminergic signaling has been explored in a variety of ways. The burst firing of dopaminergic neurons in the ventral tegmental area (VTA) is thought to reflect a novelty detection mechanism in the brain (Schultz, 1998). The hippocampus, being a major site of memory formation, is a logical location for detection of novelty. In fact, infusion of N-methyl D-aspartate (NMDA) into the ventral hippocampus (VHip) increases neuronal activity in the VTA (Lodge and Grace, 2006). Conversely, abnormalities in the hippocampus can produce abnormal dopaminergic signaling. In the methylazoxymethanol acetate (MAM) rodent model of schizophrenia, both the VHip and the VTA display abnormally high neuronal activity under baseline conditions. When the VHip is silenced with tetrodotoxin, the firing rates of neurons in the VTA are normalized (Lodge and Grace, 2007). Thus, abnormalities in dopaminergic signaling observed in schizophrenia could be related to abnormalities in the hippocampus, which are also known to occur in the disease (Harrison, 2004).

The hippocampus not only modulates, but also responds to dopaminergic activity. Dopamine is released in the hippocampus following exposure to a novel environment (Ihalainen et al., 1999) and it influences hippocampal-dependent learning (Gasbarri et al.,

1996). Dopamine application selectively depresses direct cortical input to area CA1 (temporoammonic pathway), but does not significantly alter the pathway from area CA3 to CA1 (Schaffer-collateral pathway) (Otmakhova and Lisman 1999). In addition, dopamine imposes a high-pass filter on signal propagation at temporoammonic-CA1 synapses, such that low frequency stimulation is attenuated and high-frequency stimulation is potentiatied (Ito and Schuman, 2007).

Here we report the characterization of several hippocampal-dependent behaviors that have not previously been studied in the poly(I:C) MIA model. We also use slice electrophysiology to characterize hippocampal responses to dopamine in this model.

Methods

Production of animals

Animals were bred and produced as previously described (Smith 2007). Briefly, pregnant females were injected i.p. with 20 mg/kg of poly(I:C) or saline on E12.5, and offspring were born and raised by the mother. After weaning at three weeks, offspring were housed with 4-5 animals of the same sex and in the same treatment group.

Behavior testing

At eight weeks of age, mice were initially tested for latent inhibition as previously described (Smith et al., 2007) to confirm that they displayed the behavioral abnormalities associated with MIA. They were then tested in the Morris water maze. In the initial session, mice were placed in the water maze with a visible platform 0.5 cm above the water marked by a black flag to familiarize the mice with the task and to confirm that

both groups were able to see the platform and swim to it at similar rates. The mice were then given four trials per day for five days with a platform hidden under the water, with a ten-minute inter-trail interval to prevent hyperthermia. Trials began when the mouse was placed in the water, in a random spot in the maze, and ended either when the mouse found the platform or when 90 sec had passed. The first and last trials of the week were probe trials with no platform present. The time that it took the mouse to find the platform in each trial was noted, and the probe trials were analyzed with Ethovision (Noldus), quantifying time spent in the target quadrant and swim speed.

Mice were also tested in a novel object recognition and object location paradigm (Fig. 1E). Mice were adapted to an open field for two, five-minute sessions on day 1. On day 2, mice were placed in the open field with two different, novel objects for five minutes. Mice were then removed, and the location of one of the two objects (the target object) was changed. After five minutes, mice were again placed in the box. On the following day, the same procedure was repeated with new objects, except instead of moving the target object, the target object was replaced with a novel target object in the same location in the second trial. The total number of nose-pokes to each object was recorded in each trial. The five objects used were randomized so that all mice saw different combinations of objects, and post-hoc analysis showed no innate preference for any of the objects (data not shown).

Finally, mice were tested for amphetamine-induced locomotion, as previously described (Ozawa et al., 2006). Mice were injected with 2.5 mg/kg of D-amphetamine (Sigma) i.p., freshly dissolved in sterile saline. Locomotion in a 50 x 50 cm open field

was recorded for 30 minutes before and 90 minutes after injection, and total distance traveled in each 10-minute bin was quantified using Ethovision.

Electrophysiology

Slices were prepared from 7 to 10 week old animals and microdissected to isolate the TA pathway, as described previously (Dvorak-Carbone and Schuman, 1999). In brief, a vibrating microtome (Leica VT1000S) was used to cut hippocampal slices (400 μm thickness) in ice-cold oxygenated artificial cerebrospinal fluid (ACSF) containing (in mM) 119 NaCl, 2.5 KCl, 1.3 $MgSO_4$, 2.5 CaCl, 1.0 NaH_2PO_4, 26.2 $NaHCO_3$, 11.0 glucose. Slices were incubated at room temperature for at least 1 hour in an interface chamber, and transferred to a submerged recording chamber perfused with ACSF at 24.5–25.5°C. For the extracellular field recordings, the dentate gyrus and CA3 were removed to eliminate the possible activation of the trisynaptic pathway or perforant path projection to area CA3. Concentric bipolar tungsten electrodes (FHC) and stimulus isolators (Axon Instruments) were used for the stimulation. Whole-cell voltage-clamp recordings from CA1 pyramidal neurons were made without visualization with an Axopatch 200B (Axon Instruments). Internal solution of patch pipettes was (in mM) 115 potassium gluconate, 20 KCl, 10 sodium phosphocreatine, 10 HEPES, 2 MgATP, 0.3 NaGTP (pH 7.3). Membrane voltage was clamped at _70 mV (without liquid junctional potential correction, absolute value of holding current < 100 pA in all recordings). Series resistance was left uncompensated. Recordings were discarded when the series resistance was over 20 M_ or either series or membrane resistance changed more than 20% during data acquisition. Data were collected by a custom program using the LabView data

acquisition system (National Instruments) for extracellular recordings, or DigiData 1200 and pClamp 9 (Axon Instruments) for intracellular recordings.

Statistical analysis

For the Morris water maze, two-way ANOVA with within-subject matching for successive time points was used, with maternal treatment and trial number the main variables. For all other experiments, Student's t-test was used for comparisons between two groups, and one-way ANOVA was used for comparisons among multiple groups, using Prizm 4.0 software (Graphpad). All data are expressed as mean ± SEM.

Preliminary Results

Behavior

In the Morris water maze, the latency to find the platform on each successive training day decreases as the mice learn the location of the hidden platform. Adult mice born to Poly(I:C)-treated mothers learned the task more quickly than controls, as indicated by a shorter latency to find the platform (Fig. 1A). Two-way ANOVA with treatment and session as variables reveals a significant main effect of session ($p < 0.0001$), and a significant effect of treatment ($p < 0.02$). Bonferroni post-hoc analysis reveals a significantly shorter latency in session 2 ($p < 0.05$), suggesting that early in training, the poly(I:C) offspring are better at learning the location of the hidden platform. However, in the probe trial at the completion of training, both groups show a similar, significant learned preference for the target quadrant, which is not present before training (Fig. 1B). Two-way ANOVA with treatment and quadrant as variables reveals a significant effect of quadrant ($p < 0.0001$) and no effect of treatment. Finally, both

groups of mice learn to swim to a visible platform at similar rates (Fig. 1C) and both groups swim at similar speeds in all probe trails (data not shown), confirming that the differences in water maze ability are not due to swimming ability or vision.

Next, working memory was tested using a novel object/novel location paradigm (Fig. 1D). In the novel location test, one of two objects in an open field arena is moved in the five minute interval between trials one and two, which causes the mice to spend more time exploring the moved (target) object in trial two. Both control and poly(I:C) offspring show a significant preference for the target object in trial 2, compared to trial 1 (* $p < 0.05$, Fig. 1E). Similarly, in the novel object test, in which one of two objects is changed in the interval between trail one and two, both groups of mice show a significant preference for the novel object in trial 2 compared to trial 1 ($p < 0.05$) (Fig. 1E). Further, compared to control offspring, poly(I:C) offspring show a stronger preference for the novel object in trial two ($p < 0.02$), suggesting a stronger preference for novelty. The objects (a suction-cup ball, a plastic pickle, a small knife, a candle and top) and locations (northwest, southwest, ect.) were randomized to account for any potential innate preferences for objects or locations, and post-hoc analysis indicated that the mice show no innate preference for any of the objects. Thus, in these two hippocampal-dependent tasks, compared to controls, the MIA offspring display evidence of enhanced learning ability.

Electrophysiology

As assayed by recording from CA1 pyramidal neurons in hippocampal slices, the offspring of poly(I:C)-treated mice produce miniature excitatory synaptic currents

(mEPSCs) that are significantly higher in amplitude and significantly less frequent than controls (Fig. 2 A,B). Higher amplitude in mEPSCs implies a change in excitatory synaptic transmission in these animals, and a lower frequency suggests either presynaptic terminal dysfunction or a reduced number of excitatory synapses in MIA offspring. On the other hand, the MIA offspring exhibit normal long-term potentiation at Schaffer collateral synapses (Fig. 2C). When dopamine (DA) is applied to slices, we observe a depression of field EPSPs (fEPSPs) evoked by TA pathway stimulation in both groups, however the amount of depression is larger in MIA offspring (Fig 2D). To assess the sensitivity fEPSP depression to DA, we applied the transmitter at increasing doses over five minute intervals. The MIA offspring display a significant enhancement of fEPSP depression at TA-CA1 synapses at each DA concentration, suggesting a hyper-responsiveness to DA. Thus, the hippocampus of MIA offspring exhibit changes in excitatory transmission, with presynaptic alterations, and a hypersensitivity to DA.

Discussion and Future Work

Abnormal DA signaling has long been considered central to schizophrenia. The affinity of anti-psychotic drugs for the D2 receptor correlates with the clinical efficacy of the drugs, and the psychomimetic drug amphetamine targets the DA system and can cause schizophrenia-like behaviors in normal subjects (Seeman et al., 1976; Kapur and Mamo, 2003). Moreover, both *in vivo* and post-mortem studies suggest abnormal DA signaling in schizophrenic brains (Abi-Dargham et al., 2000; Guillin et al., 2007; Kegeles et al., 2008). Using a mouse model with high face and construct value for schizophrenia, we demonstrate abnormal DA sensitivity in the hippocampus as well as abnormal hippocampal-dependent behaviors.

Our results indicate that some of the behavioral abnormalities that we observe in the offspring of poly(I:C)-treated mice may be due to hypersensivity to DA in the hippocampus. Upon introduction to a novel environment or situation, dopaminergic neurons in the VTA fire in bursts, which transiently elevates the concentration of DA in many key brain areas, leading to increased arousal (Schultz, 1998; Lodge and Grace, 2006). In the hippocampus, this elevation of DA acts as a high-pass filter for neurons in the TA pathway (Ito and Schuman, 2007), likely amplifying signals that are relevant to the novel stimulus. In the adult offspring of poly(I:C)-treated animals, the hippocampal sensitivity to DA is increased, which could result in activating the high-pass filter at lower DA levels. Functionally, this could improve the animal's performance in hippocampal-dependent behaviors,. In fact, we find that the offspring of poly(I:C)-treated mothers learn faster in water maze probe trials, and show increased novelty discrimination in the object-switching test. Taken together, these results suggest that hypersensitivity to DA may be a key mechanism in the abnormal behavior of the offspring of poly(I:C)-treated mothers.

Future behavioral work will examine the effect of the DA-releaser amphetamine on locomotive behavior. Our current results suggest the possibility of behavioral hyper-sensitivity to amphetamine, if the hippocampus results are generalizable to other brain areas. Further electrophysiology work includes confirmation of preliminary data (Fig. 2), and examination of the response to a single concentration of DA over time. It will also be of interest to examine DA receptor expression in the hippocampus in an effort to begin to explore the basis of the apparent DA hypersensitivity.

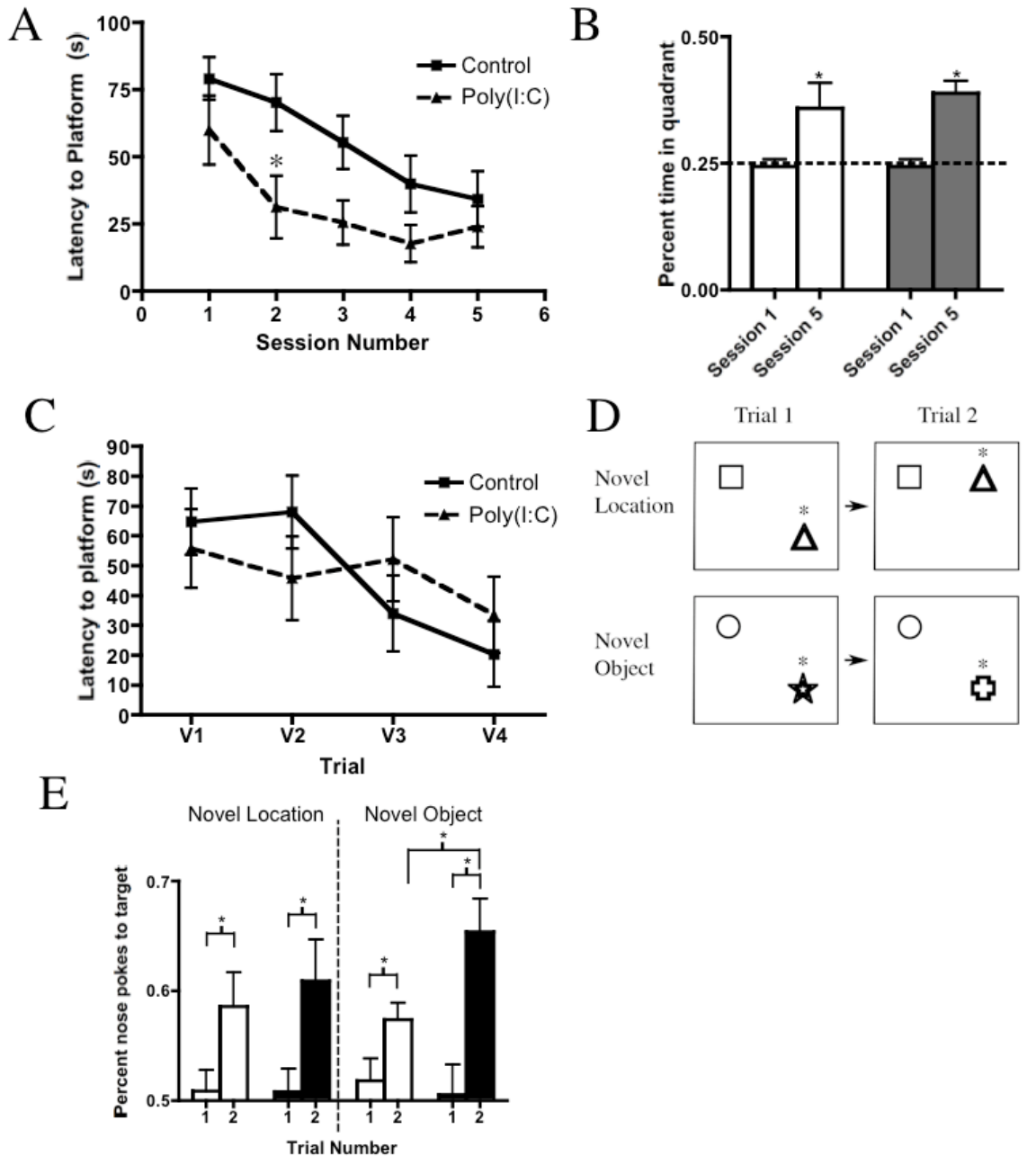


Figure 1. Hippocampal-dependent behaviors in the adult offspring of poly(I:C)-treated mothers. (A) In the Morris water maze, the latency to find the platform is significantly shorter in the MIA offspring (dashed line) compared to controls (solid line). Two-way ANOVA with treatment and session as variables reveals a significant main

effect of session ($p < 0.0001$), and a significant effect of treatment ($p < 0.02$). In session 2, the MIA offspring had a significantly shorter latency to find the platform * $p < 0.05$ (B) Both control (open bars) and MIA offspring (filled bars) show a significant learned preference for the target quadrant in the session 6 probe trial, which was not present before training. * $p < 0.05$ vs session 1. (C) Both groups were able to find the platform during the initial platform-visable task, indicating similar ability to swim to the platform. (D) A graphical representation of the novel place and novel object tests illustrates how the location of the target object, or the target object itself, is changed in the 5 minute interval between trial 1 and 2. Asterisks indicate the target object. (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 (* $p < 0.05$). Similarly, in the novel object test, both groups 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.

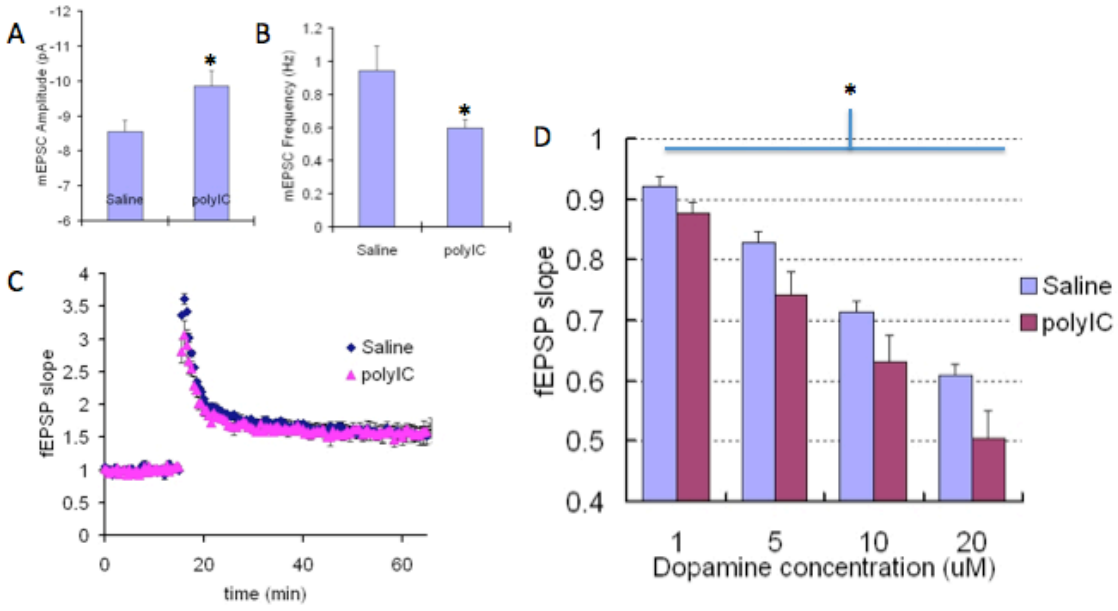


Figure 2. Offspring of poly(I:C)-injected mothers are hyper-responsive to dopamine. CA1 neurons in slices prepared from MIA offspring have increased amplitude (A) and decreased frequency (B) of miniature EPSCs. Induction of LTP at Schaffer collateral synapses is normal (C). However, when the neurons are exposed to increasing concentrations of DA, neurons from MIA offspring are hyper-responsive, displaying a decreased EPSP slope compared to control neurons. * $p < 0.05$

References

Abi-Dargham A (2004) Do we still believe in the dopamine hypothesis? New data bring new evidence. Int J Neuropsychopharmacol 7 Suppl 1:S1-5.

Abi-Dargham A, Rodenhiser J, Printz D, Zea-Ponce Y, Gil R, Kegeles LS, Weiss R, Cooper TB, Mann JJ, Van Heertum RL, Gorman JM, Laruelle M (2000) Increased baseline occupancy of D2 receptors by dopamine in schizophrenia. Proc Natl Acad Sci U S A 97:8104-8109.

Babulas V, Factor-Litvak P, Goetz R, Schaefer CA, Brown AS (2006) Prenatal exposure to maternal genital and reproductive infections and adult schizophrenia. Am J Psychiatry 163:927-929.

Bakos J, Duncko R, Makatsori A, Pirnik Z, Kiss A, Jezova D (2004) Prenatal immune challenge affects growth, behavior, and brain dopamine in offspring. Ann N Y Acad Sci 1018:281-287.

Brown AS (2006) Prenatal infection as a risk factor for schizophrenia. Schizophr Bull 32:200-202.

Brown AS, Susser ES (2002) In utero infection and adult schizophrenia. Ment Retard Dev Disabil Res Rev 8:51-57.

Brown AS, Schaefer CA, Quesenberry CP, Jr., Liu L, Babulas VP, Susser ES (2005) Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. Am J Psychiatry 162:767-773.

Brown AS, Begg MD, Gravenstein S, Schaefer CA, Wyatt RJ, Bresnahan M, Babulas VP, Susser ES (2004a) Serologic evidence of prenatal influenza in the etiology of schizophrenia. Arch Gen Psychiatry 61:774-780.

Brown AS, Hooton J, Schaefer CA, Zhang H, Petkova E, Babulas V, Perrin M, Gorman JM, Susser ES (2004b) Elevated maternal interleukin-8 levels and risk of schizophrenia in adult offspring. Am J Psychiatry 161:889-895.

Chess S (1977) Follow-up report on autism in congenital rubella. J Autism Child Schizophr 7:69-81.

Ciaranello AL, Ciaranello RD (1995) The neurobiology of infantile autism. Annu Rev Neurosci 18:101-128.

Desmond MM, Wilson GS, Melnick JL, Singer DB, Zion TE, Rudolph AJ, Pineda RG, Ziai MH, Blattner RJ (1967) Congenital rubella encephalitis. Course and early sequelae. J Pediatr 71:311-331.

Dvorak-Carbone H, Schuman EM (1999) Long-term depression of temporoammonic-CA1 hippocampal synaptic transmission. J Neurophysiol 81:1036-1044.

Fortier ME, Joober R, Luheshi GN, Boksa P (2004a) Maternal exposure to bacterial endotoxin during pregnancy enhances amphetamine-induced locomotion and startle responses in adult rat offspring. J Psychiatr Res 38:335-345.

Fortier ME, Kent S, Ashdown H, Poole S, Boksa P, Luheshi GN (2004b) The viral mimic, polyinosinic:polycytidylic acid, induces fever in rats via an interleukin-1-dependent mechanism. Am J Physiol Regul Integr Comp Physiol 287:R759-766.

Gasbarri A, Sulli A, Innocenzi R, Pacitti C, Brioni JD (1996) Spatial memory impairment induced by lesion of the mesohippocampal dopaminergic system in the rat. Neuroscience 74:1037-1044.

Golan HM, Lev V, Hallak M, Sorokin Y, Huleihel M (2005) Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy. Neuropharmacology 48:903-917.

Guillin O, Abi-Dargham A, Laruelle M (2007) Neurobiology of dopamine in schizophrenia. Int Rev Neurobiol 78:1-39.

Harrison PJ (2004) The hippocampus in schizophrenia: a review of the neuropathological evidence and its pathophysiological implications. Psychopharmacology (Berl) 174:151-162.

Hava G, Vered L, Yael M, Mordechai H, Mahoud H (2006) Alterations in behavior in adult offspring mice following maternal inflammation during pregnancy. Dev Psychobiol 48:162-168.

Hyman SL, Arndt TL, Rodier PM (2005) Environmental Agents and Autism: Once and Future Associations. International Review of Research in Mental Retardation Volume 30:171-194.

Ihalainen JA, Riekkinen P, Jr., Feenstra MG (1999) Comparison of dopamine and noradrenaline release in mouse prefrontal cortex, striatum and hippocampus using microdialysis. Neurosci Lett 277:71-74.

Ito HT, Schuman EM (2007) Frequency-dependent gating of synaptic transmission and plasticity by dopamine. Front Neural Circuits 1.

Kapur S, Mamo D (2003) Half a century of antipsychotics and still a central role for dopamine D2 receptors. Prog Neuropsychopharmacol Biol Psychiatry 27:1081-1090.

Kegeles LS, Slifstein M, Frankle WG, Xu X, Hackett E, Bae SA, Gonzales R, Kim JH, Alvarez B, Gil R, Laruelle M, Abi-Dargham A (2008) Dose-Occupancy Study of Striatal and Extrastriatal Dopamine D(2) Receptors by Aripiprazole in Schizophrenia with PET and [(18)F]Fallypride. Neuropsychopharmacology.

Libbey JE, Sweeten TL, McMahon WM, Fujinami RS (2005) Autistic disorder and viral infections. J Neurovirol 11:1-10.

Lodge DJ, Grace AA (2006) The hippocampus modulates dopamine neuron responsivity by regulating the intensity of phasic neuron activation. Neuropsychopharmacology 31:1356-1361.

Lodge DJ, Grace AA (2007) Aberrant hippocampal activity underlies the dopamine dysregulation in an animal model of schizophrenia. J Neurosci 27:11424-11430.

Meisenzahl EM, Schmitt GJ, Scheuerecker J, Moller HJ (2007) The role of dopamine for the pathophysiology of schizophrenia. Int Rev Psychiatry 19:337-345.

Nyffeler M, Meyer U, Yee BK, Feldon J, Knuesel I (2006) Maternal immune activation during pregnancy increases limbic GABAA receptor immunoreactivity in the adult offspring: implications for schizophrenia. Neuroscience 143:51-62.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Patterson PH (2005) Maternal influenza infection leads to neuropathology and behavioral abnormalities in adult offspring. Neuropsychopharmacology 30:S9-S9.

Schultz W (1998) Predictive reward signal of dopamine neurons. J Neurophysiol 80:1-27.

Seeman P, Lee T, Chau-Wong M, Wong K (1976) Antipsychotic drug doses and neuroleptic/dopamine receptors. Nature 261:717-719.

Shi L, Fatemi SH, Sidwell RW, Patterson PH (2003) Maternal influenza infection causes marked behavioral and pharmacological changes in the offspring. J Neurosci 23:297-302.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Appendix A

Double-Hit and Tail-Vein Injection Models of MIA:

Attempting to Induce Inflammation in the Adult Brain

Stephen Smith, Hae Jin Kang, Jennifer Li and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

Rationale

Recent data indicates that both schizophrenia and autism are characterized by immune abnormalities, both in the periphery and in the brain. Several studies have shown elevated levels of cytokines in the blood and cerebrospinal fluid (CSF) of schizophrenic (Garver et al., 2003; Zhang et al., 2005) and autistic patients (Singh et al., 1991; Croonenberghs et al., 2002; Zimmerman et al., 2005; Ashwood et al., 2006; Chez et al., 2007). A recent microarray study also showed upregulation of immune-related genes in post-mortem autistic brain tissue (Garbett et al., 2007). Furthermore, severe inflammation was seen in post-mortem autistic brains, characterized by high levels of inflammatory chemokines and cytokines, as well as astrogliosis and microgliosis (Vargas et al., 2005). A replication of the severe microgliosis was recently presented (Morgan et al., 2007). The large age range of patients (3-50 yrs) as well as the diverse autism sub-types (regressive and non-regressive; seizures present or absent) that displayed this inflammation suggests that it may be a central feature of the autistic phenotype.

The observation that autistic symptoms seem to improve during episodes of fever (Curran et al., 2007) also highlights the interconnectivity of the immune and nervous systems. In fact, because inflammatory cytokines can regulate neuronal excitability, long-term potentiation and learning, the stress response, feeding, sleep and depressive behaviors (Jankowsky and Patterson, 1999; Capuron and Dantzer, 2003; Balschun et al., 2004; Theoharides et al., 2004; Schiepers et al., 2005; Bauer et al., 2007), it is possible that immunological dysfunction in the brain is the direct cause of certain autism-related behaviors. Because of the efficacy, safety and availability of anti-inflammatory drugs, this observation offers a potential treatment strategy. In fact, a small pilot study using the

anti-inflammatory drug pioglitazone showed promising results in autistic children (Boris et al., 2007), and a larger clinical trial of minocycline is currently underway at the NIH.

Because of the potential role of inflammation in both the pathogenesis and treatment of the disease, we set out to observe cellular inflammation in our animal model of autism and schizophrenia, the maternal immune activation (MIA) model. Prior work with both the poly(I:C) and LPS models indicate that more severe treatments are associated with immune dysregulation in the offspring (for discussion, see Chapter 2). The most severe published treatement, in which pregnant female rats were injected with LPS every day throught pregnancy, resulted in high levels of inflammatory cytokines in adult offspring that had never been exposed to LPS postnatally (Romero et al., 2007). Results from labs that use tail vein injection of poly(I:C) to induce MIA indicate that behavioral markers are at least as robust as in our model, even thought these groups inject only 4-5 mg/kg of poly(I:C), while we use 20 mg/kg I.P. (Zuckerman et al., 2003; Zuckerman and Weiner, 2005; Meyer et al., 2006; Nyffeler et al., 2006; Ozawa et al., 2006; Meyer et al., 2007). Further, one group has reported pyknotic cells in the hippocampus (Zuckerman et al., 2003), although this phenotype was not seen elsewhere (Meyer et al., 2007).

Preliminary Studies

We began by assaying brains for molecular markers of inflammation, similar to those reported by Vargas et al. (2005). In that paper, IL-6 was elevated in both the cortex and hippocampus, as measured by a Ray-Biotech protein multi-array, which is a semi-quantitative antibody-sandwich assay. We performed the mouse version of this assay

(Ray-Biotech mouse inflammation array containing 40 cytokines, for list see Chapter 3 Fig. S2) on adult brain extracts from mice whose mothers were injected with poly(I:C) or saline on E12.5. We were unable to identify differences between the two groups (data not shown), so we utilized the more sensitive ELISA and focused on one of the most up-regulated cytokines in the Vargas study, IL-6. Although preliminary data showed significantly higher levels of IL-6 in the cerebellum, and a trend towards significantly higher levels IL-6 in the hippocampus of MIA offspring, when we repeated the experiment with a larger number of samples and a more sensitive standard curve that was more appropriate for the low levels of IL-6 that we were detecting, we found no difference in IL-6 levels between the two groups (Fig1a,b). Morevoer, Western blot analysis of whole brain extracts provided no evidence of astrogliosis in the hippocampus or cerebellum (Fig. 1c,d), and immunoflorescence staining of adult brain sections from MIA offspring and controls showed no obvious difference in the levels of astrogliosis (GFAP, 1:1000) or microgliosis (Mac1, 1:200) (data not shown).

We then set out to establish a mouse model that does show signs of inflammation in the adult brain. We focused on two treatments that are more severe than our usual method of intraperitoneal injection of poly(I:C) in the pregnant mouse: tail vein (i.v.) injection in the pregnant mouse, and a "double-hit" model in which the offspring of an E12.5 poly(I:C)-injected female are injected with poly(I:C) as pups. The rationale behind the i.v. model is that, because the poly(I:C) enters the blood stream and directly interacts with the placenta, the spleen, and other major maternal organs, there is the potential for a more severe reaction than with i.p. injection, where most of the poly(I:C) is likely taken up by peritoneal macrophages. For example, the placenta expresses the poly(I:C)

receptor TLR-3, and thus may respond more severely to i.v. administration (Nishimura and Naito, 2005). In fact, several studies using i.v. injection use less poly(I:C) than we inject i.p. (4-5 mg/kg vs. 20 mg/kg) and some have reported more severe results in the offspring, such as inflammation in the adult brain (Zuckerman et al., 2003; Zuckerman and Weiner, 2005; Ozawa et al., 2006). In addition, a very severe protocol of daily LPS injection results in markedly elevated cytokine levels in the serum of the adult offspring (Romero et al., 2007) suggesting an ongoing inflammatory reaction.

The rationale for using the double-hit model stems from several studies in rodents indicating that infection of very young animals can lead to an abnormal immune responses later in life (Boisse et al., 2004; Bilbo et al., 2005b; Bilbo et al., 2005a). Moreover, there anecdotal reports of autism appearing after sickness or vaccination of the child (e.g., (Poling et al., 2006)). We hypothesize that the initial maternal immune activation may prime the offspring's immune system such that when it is exposed to poly(I:C) early in development, we could trigger inflammation and behavioral changes such as those reported by Vargas et al. (2005).

For the i.v. injection model, we injected mice with 4, 10 or 15 mg/kg of poly(I:C) (or saline) via the tail vein, under mild physical restraint. Offspring were weaned as normal, and behaviorally tested beginning at 8 weeks. All i.v. poly(I:C) offspring showed significant reductions in PPI (Fig. 2A,B). However, the other behavioral assays did not show significant effects of treatment group. In the latent inhibition (LI) test (Fig. 2C), the offspring of mice treated with the highest doses (10 and 15 mg/kg) of poly(I:C) show no LI, as indicated by freezing rates that were close to the not-pre-exposed (NPE) animals. The control mice and the mice treated with 4 mg/kg show a trend towards

significant LI, but none of these effects reached statistical significance. A possible dose-dependent effect was seen in time spent in the center of the open field, although again, this was not significant. For 0, 4, 10 and 15 mg/kg, the N values were 30, 37, 21, 23 for the open field and PPI tests, N = 14, 20, 15, 17 for the pre-exposed LI test, and 5, 5, 3, 4 for the NPE mice. After behavioral testing, the mice were examined for signs of microglial activation. Sections were stained for Mac1 and DAPI, and Mac1+ cells were scored when the DAPI staining co-localized with Mac1+ cell body. There was a significant increase in the number of Mac1+ cells in several different regions of the brain, including the agranular retrosplenial cortex (RSA), the dentate gyrus (DG), and areas CA1 and CA3 of the hippocampus (Fig. 3). This suggests that i.v. injection may induce long-lasting inflammation in the brain. However, the behavioral results were mixed, and the level of inflammation seen in the brain was not nearly as severe as that seen in autistic brains.

To produce "double-hit" (DH) mice, pregnant females were injected with poly(I:C) on E12.5. In two separate experiments, offspring were then injected with either poly(I:C) (dose) or saline on day 3 (DH-P3) or day 7 (DH-P7). This yielded four groups per experiment, defined by maternal/offspring treatment: saline/saline (SS), saline/poly(I:C) (SP), poly(I:C)/saline (PS), and poly(I:C)/poly(I:C) (PP). Data from the DH-P3 experiment are shown in Fig. 4; N = 10, 9, 10, 15 for SS, SP, PS, PP. The behavioral testing (LI, PPI, Open Field) of this cohort was negative; 2-way ANOVAs with prenatal treatment and postnatal treatment as the main variables revealed no significant differences in any of the behavioral parameters measured. Definite conclusions were undermined by the lack of effect of prenatal MIA, however.

Nonetheless, close examination of the data reveals some potentially promising trends. For example, in the LI test, we expect SS mice to show significant LI, and to freeze for only 30-40% of the tone presentations; however, they froze for 70% and showed no LI. Similar patterns emerged for PPI (5 and 15). It should be noted that, due to the small N in both double-hit pilot experiments, an NPE group was not used, making interpretation of LI results more difficult. Histology was not performed on this group.

Data from the DH-P7 experiment is shown in Fig. 5; N = 9, 24, 11, 14 for SS, SP, PS, PP. Again, 2-way ANOVAs with prenatal treatment and postnatal treatment as the main variables revealed no significant differences in the PPI and LI tests (Fig. 5A-C). In the LI test, there seemed to be a trend towards higher freezing, and less LI in the groups that received prenatal, and double-hit, poly(I:C). Open field distance traveled showed a significant effect of post-natal treatment ($p = 0.004$), with postnatal poly(I:C) causing hyperactivity in the open field. As previously demonstrated (Smith et al., 2007), prenatal poly(I:C) causes a significant decrease in the number of entries into the open field ($P = 0.02$), while postnatal poly(I:C) caused a significant increase in the number of center entries ($p = 0.02$), consistent with the hyperactivity phenotype. These behavioral tests are also hard to interpret, because prenatal poly(I:C) only showed the expected significant effect in entries into the center of the open field, but no effect on PPI or LI. Moreover, PN poly(I:C) caused hyperactivity in the open field, not the expected heightened anxiety phenotype, and did not affect the other behaviors assayed. Brain sections were cut and stained for Mac1+ and DAPI, and both the optical density of Mac1 staining and the number of Mac1+/DAPI+ cell bodies were counted. The data in Fig. 6 show that there

was a significantly higher optical density, and a significantly higher number of $Mac1^+$ cells in the poly(I:C)/poly(I:C) mice.

Future Directions

Although both the i.v. and the DH-P7 mice did not display the expected behavioral phenotype, there was evidence of a mild cellular inflammatory reaction, i.e. microglial activation, in the brains of the adult animals. Moreover, both the i.v. and DH-P7 mice did show some significant behavioral changes due to the experimental manipulations; i.v.-injected offspring showed a significant PPI deficit and DH-P7 offspring showed alterations in open field behavior. These results are promising, particularly considering the difficulties of behavioral testing. In previous experiments, we have found that we require larger numbers of animals than we used in this pilot study to achieve statistically significant behavioral results. The fact that we were able to document increased microglia in the adult brains suggests that our hypothesis, that increased severity of early immune challenge can cause an ongoing inflammatory state in the brain, inidcates that further experiments are warranted.

We have modified our protocol to use a maternal i.v. poly(I:C) injection, followed by injection of P7 pups, to combine the i.v. and DH-P7 models. The mice will be behaviorally tested in late spring, and sections will be examined for inflammation over the summer. Should we be able to document both increased inflammation and abnormal behavior in a adult mouse model of MIA, we plan use immunosuppressive drugs, such as pioglitazone or minocycline, to determine if inflammation in the brain can be ameliorated and behavioral abnormalities corrected, with obvious treatment implications for autism.

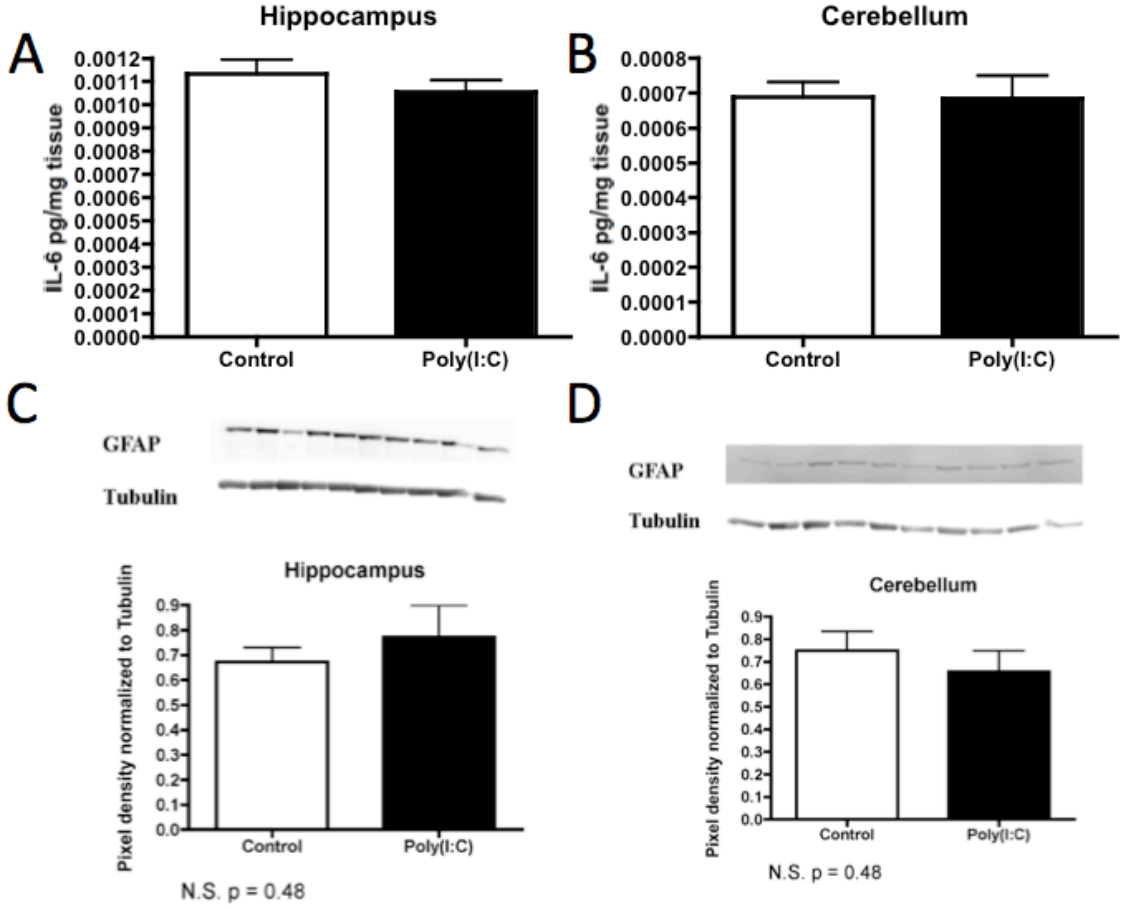


Figure 1. No signs of inflammation are present in the adult brains of MIA offspring. ELISAs for IL-6 in the hippocampus (A) or the cerebellum (B) show identical levels in control and poly(I:C) exposed offspring. Moreover, as determined by quantitative Western blotting, the levels of GFAP in the hippocampus (C) or cerebellum (D) are similar in both groups.

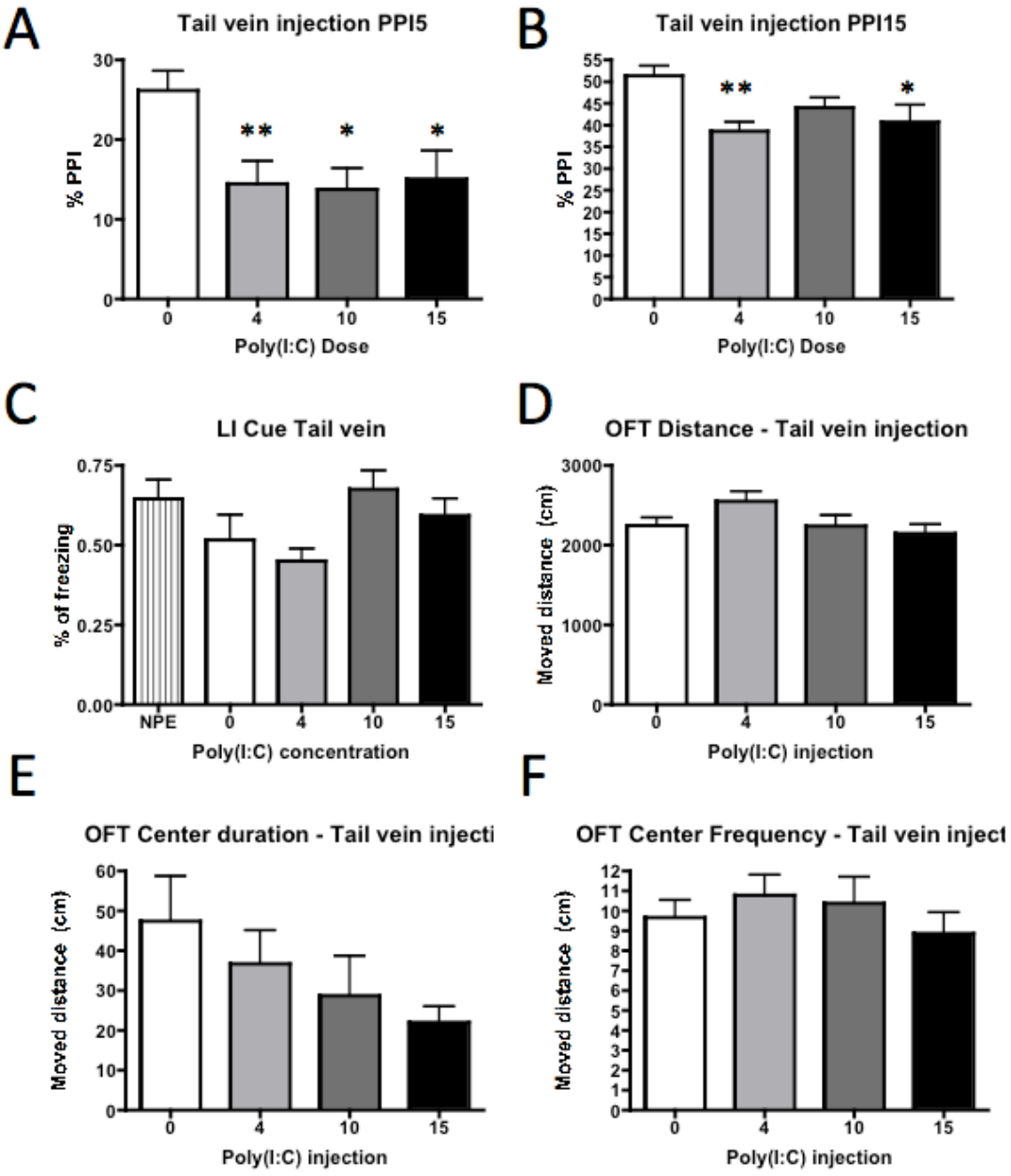


Figure 2. Behavioral analysis of the offspring of tail-vein injected mice reveals some effects of MIA. Mice showed significant reductions in PPI, both at a prepulse of 5 and 15db (A,B). In the LI paradigm, none of groups were significantly different (C); however, the 10 mg/kg and 15 mg/kg groups appear to show no LI, while the 0 and 4 mg/kg groups appear to show a trend towards LI. In the open field test, no significant

differences were found in open field distance (D), time spent in the center of the field (E) or entries into the center of the field (F). * $p < 0.05$, ** $p < 0.01$

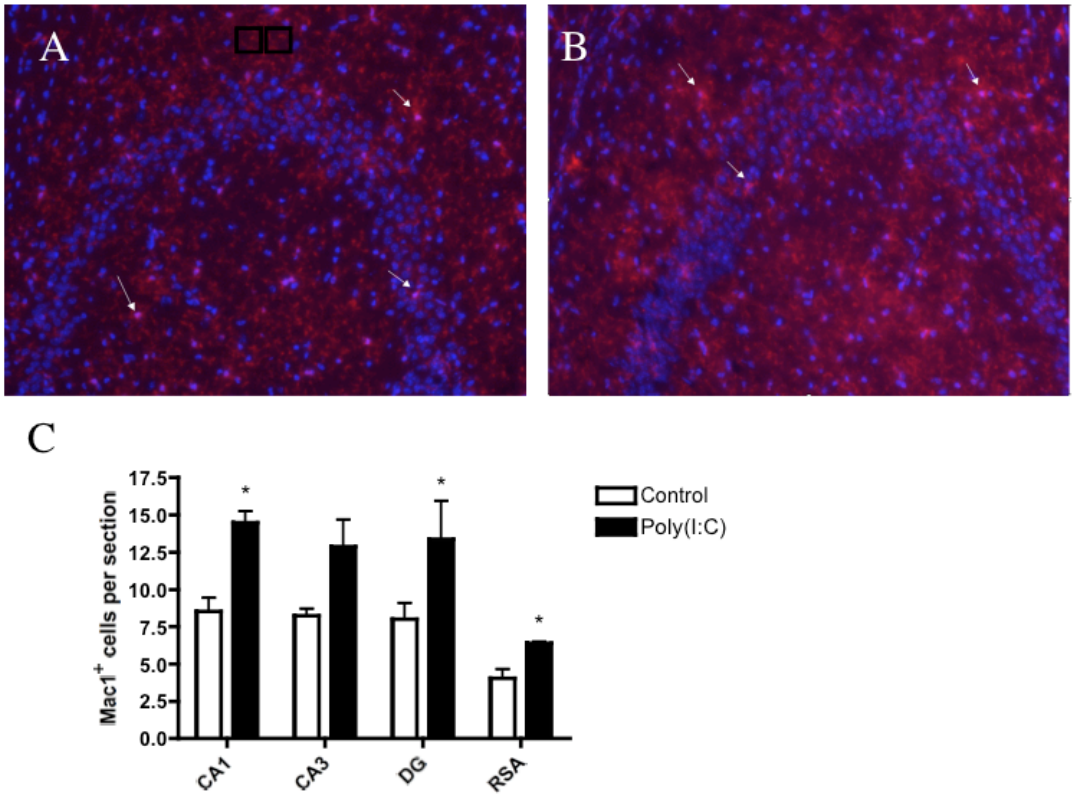


Figure 3. More microglia are found in the brains of adult poly(I:C) tail vein injected mice. Representative sections from the CA3 region of control (A) and poly(I:C) TV injected (B) adult animals showing Mac1+/DAPI+ cell bodies (arrows). (C) Quantification of sections by counting the number of Mac1+ cells per section revealed significantly higher numbers of microglia in several brain areas of poly(I:C) TV injected offspring. CA1, CA3, regions of the hippocampus; DG, dentate gyrus; RSA, agranular retrospenal cortex. * $p < 0.05$.

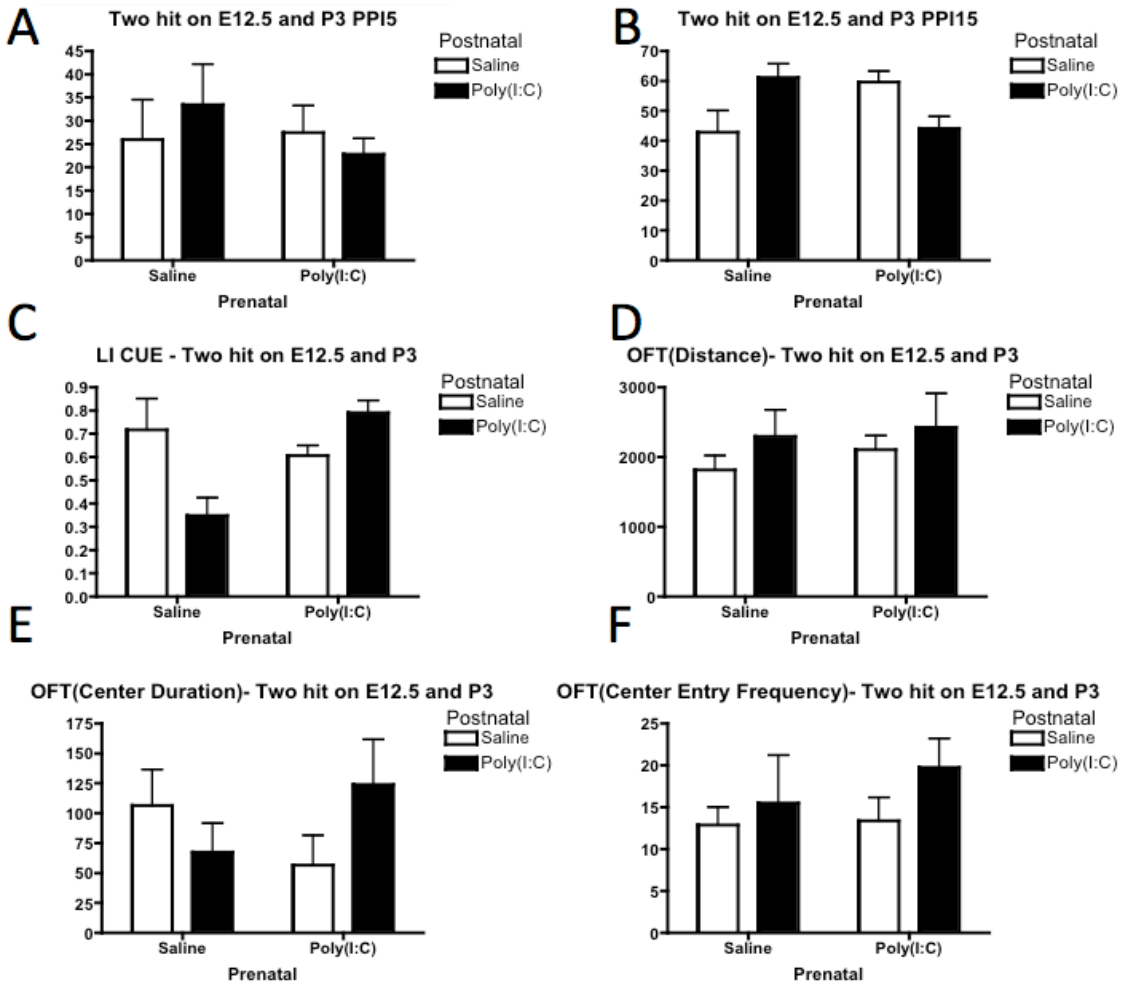


Figure 4. Behavioral analysis of DH-P3 mice reveals no significant effects on behavior. Pregnant dams were injected with 20 mg/kg poly(I:C) on E12.5, and pups were injected with 20 mg/kg poly(I:C) on P3. Two-way ANOVA reveals no effect of either prenatal or postnatal treatment for any behavior; PPI (A,B) LI (C), open field distance traveled (D), time spent in the center (E) or entries into the center (F).

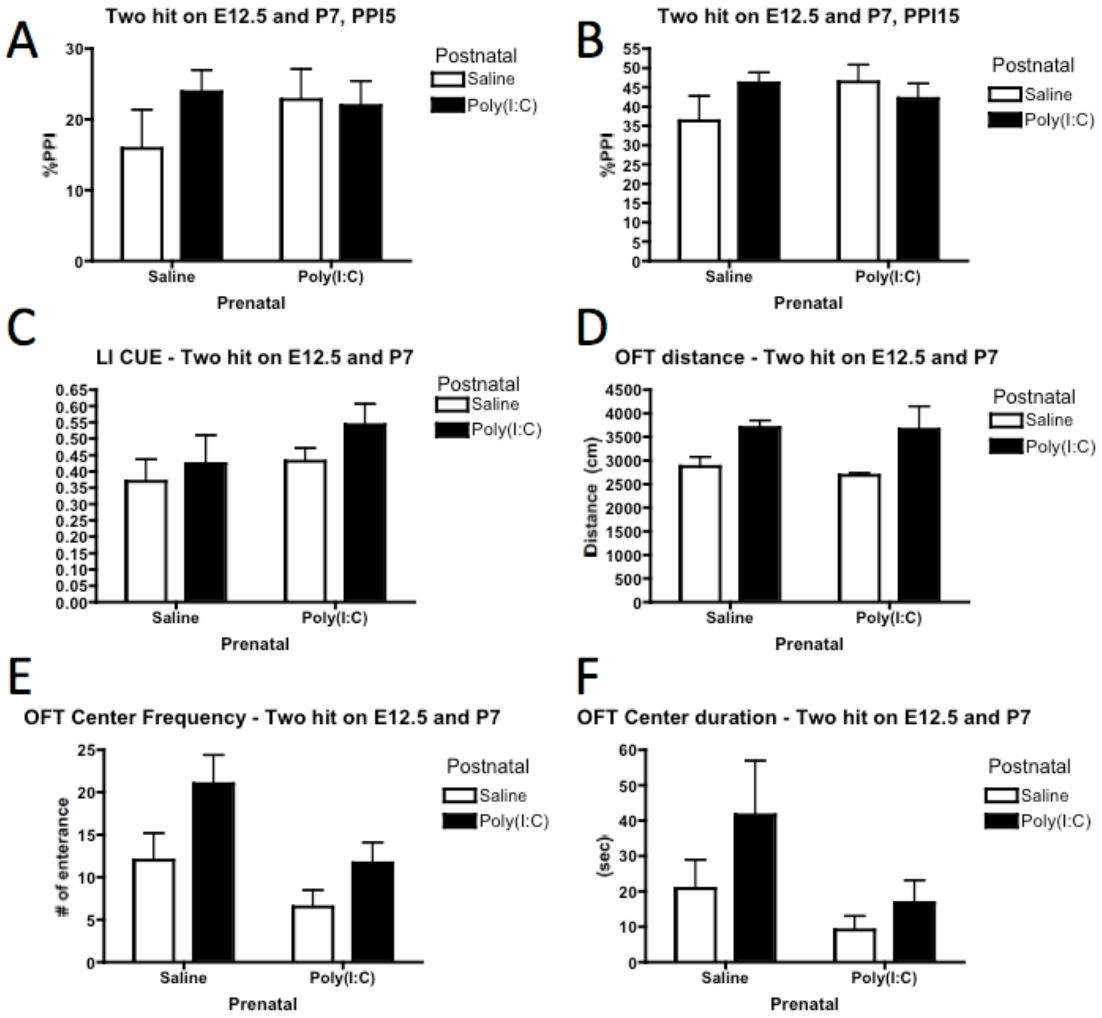


Figure 5. Behavioral analysis of DH-P7 animals reveals some effects of double-hit treatment. Pregnant dams were injected with 20 mg/kg of poly(I:C) on E12.5, and pups were undetected with 20 mg/kg poly(I:C) on P7. Two-way ANOVA reveals a significant effect of postnatal treatment on open field distance (D) and a significant effect of both prenatal and postnatal treatment for open field center entries (E). All other comparisons are not significantly different.

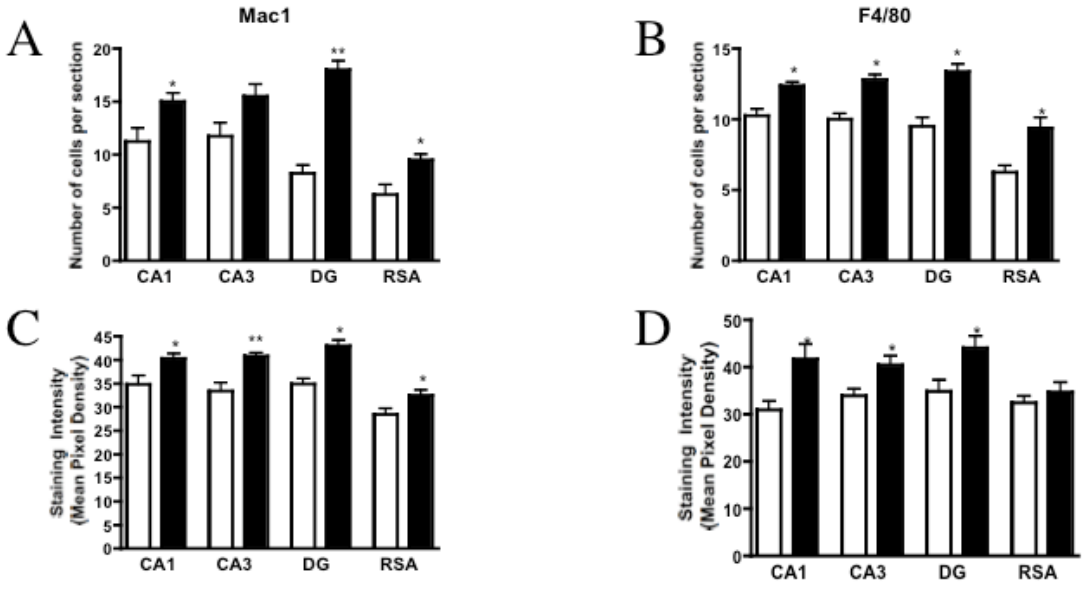


Figure 6: DH-P7 mice have increased numbers of microglia in the adult brain. Staining was performed as in Fig. 3. Two methods of quantification of staining for two different microglial antigens, Mac1 and F4/80, revealed significantly higher numbers of microglia (A,B) and significantly higher amount of staining measured by pixel density (C,D) in double-hit animals (black bars) compared to controls (white bars). CA1, CA3, regions of the hippocampus; DG, dentate gyrus; RSA, agranular retrospenal cortex. * $p < 0.05$; ** $p < 0.01$

References

Ashwood P, Wills S, Van de Water J (2006) The immune response in autism: a new frontier for autism research. J Leukoc Biol 80:1-15.

Balschun D, Wetzel W, Del Rey A, Pitossi F, Schneider H, Zuschratter W, Besedovsky HO (2004) Interleukin-6: a cytokine to forget. Faseb J 18:1788-1790.

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. Nat Rev Neurosci 8:221-232.

Bilbo SD, Levkoff LH, Mahoney JH, Watkins LR, Rudy JW, Maier SF (2005a) Neonatal infection induces memory impairments following an immune challenge in adulthood. Behav Neurosci 119:293-301.

Bilbo SD, Biedenkapp JC, Der-Avakian A, Watkins LR, Rudy JW, Maier SF (2005b) Neonatal infection-induced memory impairment after lipopolysaccharide in adulthood is prevented via caspase-1 inhibition. J Neurosci 25:8000-8009.

Boisse L, Mouihate A, Ellis S, Pittman QJ (2004) Long-term alterations in neuroimmune responses after neonatal exposure to lipopolysaccharide. J Neurosci 24:4928-4934.

Boris M, Kaiser CC, Goldblatt A, Elice MW, Edelson SM, Adams JB, Feinstein DL (2007) Effect of pioglitazone treatment on behavioral symptoms in autistic children. J Neuroinflammation 4:3.

Capuron L, Dantzer R (2003) Cytokines and depression: the need for a new paradigm. Brain Behav Immun 17 Suppl 1:S119-124.

Chez MG, Dowling T, Patel PB, Khanna P, Kominsky M (2007) Elevation of tumor necrosis factor-alpha in cerebrospinal fluid of autistic children. Pediatr Neurol 36:361-365.

Croonenberghs J, Bosmans E, Deboutte D, Kenis G, Maes M (2002) Activation of the inflammatory response system in autism. Neuropsychobiology 45:1-6.

Curran LK, Newschaffer CJ, Lee LC, Crawford SO, Johnston MV, Zimmerman AW (2007) Behaviors associated with fever in children with autism spectrum disorders. Pediatrics 120:e1386-1392.

Garbett K, Ebert P, Lintas C, Mirnics K, Persico A (2007) Immune transcript disturbances in temporal cortex of autistic brains. 2007 Society for Neuroscience poster presentation.

Garver DL, Tamas RL, Holcomb JA (2003) Elevated interleukin-6 in the cerebrospinal fluid of a previously delineated schizophrenia subtype. Neuropsychopharmacology 28:1515-1520.

Jankowsky JL, Patterson PH (1999) Cytokine and growth factor involvement in long-term potentiation. Mol Cell Neurosci 14:273-286.

Meyer U, Murray PJ, Urwyler A, Yee BK, Schedlowski M, Feldon J (2007) Adult behavioral and pharmacological dysfunctions following disruption of the fetal brain balance between pro-inflammatory and IL-10-mediated anti-inflammatory signaling. Mol Psychiatry.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Morgan JT, Chana G, Buckwalter J, Courchesne E, Everall IP (2007) Increased Iba-1 positive microglial cell denisty in the autistic brain. 2007 Society for Neuroscience poster presentation.

Nishimura M, Naito S (2005) Tissue-specific mRNA expression profiles of human toll-like receptors and related genes. Biol Pharm Bull 28:886-892.

Nyffeler M, Meyer U, Yee BK, Feldon J, Knuesel I (2006) Maternal immune activation during pregnancy increases limbic GABAA receptor immunoreactivity in the adult offspring: implications for schizophrenia. Neuroscience 143:51-62.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Poling JS, Frye RE, Shoffner J, Zimmerman AW (2006) Developmental regression and mitochondrial dysfunction in a child with autism. J Child Neurol 21:170-172.

Romero E, Ali C, Molina-Holgado E, Castellano B, Guaza C, Borrell J (2007) Neurobehavioral and immunological consequences of prenatal immune activation in rats. Influence of antipsychotics. Neuropsychopharmacology 32:1791-1804.

Schiepers OJ, Wichers MC, Maes M (2005) Cytokines and major depression. Prog Neuropsychopharmacol Biol Psychiatry 29:201-217.

Singh VK, Warren RP, Odell JD, Cole P (1991) Changes of soluble interleukin-2, interleukin-2 receptor, T8 antigen, and interleukin-1 in the serum of autistic children. Clin Immunol Immunopathol 61:448-455.

Theoharides TC, Weinkauf C, Conti P (2004) Brain cytokines and neuropsychiatric disorders. J Clin Psychopharmacol 24:577-581.

Vargas DL, Nascimbene C, Krishnan C, Zimmerman AW, Pardo CA (2005) Neuroglial activation and neuroinflammation in the brain of patients with autism. Ann Neurol 57:67-81.

Zhang XY, Zhou DF, Cao LY, Wu GY, Shen YC (2005) Cortisol and cytokines in chronic and treatment-resistant patients with schizophrenia: association with psychopathology and response to antipsychotics. Neuropsychopharmacology 30:1532-1538.

Zimmerman AW, Jyonouchi H, Comi AM, Connors SL, Milstien S, Varsou A, Heyes MP (2005) Cerebrospinal fluid and serum markers of inflammation in autism. Pediatr Neurol 33:195-201.

Zuckerman L, Weiner I (2005) Maternal immune activation leads to behavioral and pharmacological changes in the adult offspring. J Psychiatr Res 39:311-323.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition,

dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Appendix B

Cellular and Molecular Permeability of the Placenta Following Maternal Immune Activation

Stephen Smith, Ilana Goldflam and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

Rationale

The observation that IL-6 is central to the maternal immune activation (MIA) model of schizophrenia and autism (Smith et al., 2007) led to the question of where IL-6 is acting. IL-6 has recently been shown to cross the placenta *in vivo* in rats (Dahlgren et al., 2006) and to cross the perfused post-partum human placenta (Zaretsky et al., 2004). These observations suggest that IL-6 it is likely able to directly interact with the fetal brain and cause changes in cellular development (for discussion see Chapters 2 and 3). However, IL-6 could also alter placental properties, or could stimulate the maternal immune system to mount a cellular response against the fetus. If IL-6 were to alter the passage of nutrients or metabolites across the placenta, fetal malnutrition or growth restriction could result, and malnutrition is a known risk factor for schizophrenia (Susser and Lin, 1992; Susser et al., 1996; van Os, 1997; Neugebauer, 2005). Another idea stems from the observation that the pregnant immune system is immuno-surpressed, possibly to prevent allogenic rejection of the fetus (Sargent et al., 2006). Perhaps IL-6 can alter that suppression and cause a decrease in tolerance of the fetus (Debnath and Chaudhuri, 2006), allowing cells to cross the placenta and attack the fetus directly. In fact, maternal-fetal, bidirectional transmission of cells is well known. In normal human pregnancy, maternal cells are detectable in 20% of second-trimester fetuses (Lo et al., 1998) and in 100% of third-trimester fetuses (Petit et al., 1997). In mice, maternal cell trafficking begins between E8.5 and E12 (Piotrowski and Croy, 1996), with maternal cells crossing into the fetus via both the placenta as well as post-natally through the milk (Arvola et al., 2000; Zhou et al., 2000; Su et al., 2008). Antibody-secreting B cells of maternal origin can be found in 5-month old B-cell deficent ($\mu^{-/-}$) mice born to $\mu^{+/-}$ mothers (Arvola et al.,

2000), and IL-2 secreting cells of maternal origin are found in adult IL-2 knockout mice (Wrenshall et al., 2007). In the context of disease, cells of maternal origin have been found at sites of auto-immune reactions in humans, including juvenile idiopathic myopathy (Artlett et al., 2000), neonatal lupus congenital heart block (Stevens et al., 2003), and Pityriasis Lichenoides (Khosrotehrani et al., 2006) (for review see (Stevens, 2007)). An intriguing hypothesis, given the immune dysregulation (see Appendix A) and higher incidence of autoimmune disease in schizophrenia and autism (Torrey and Yolken, 2001; Jones et al., 2005; Ashwood et al., 2006; Pardo and Eberhart, 2007), is that maternal cells persist in the offspring's brain and stimulate an auto-immune response, the side-effects of which can cause psychiatric symptoms. If maternal cells, activated by IL-6, do cross into the fetus in greater numbers or migrate to the brain, it could suggest either a direct immune-mediated attack of the fetal brain, the release of cytokines detrimental to fetal brain development, or it could lead to a an auto-immune response later in life. Therefore, we began to characterize the response of the placenta to MIA, and to determine if we could detect a change in the number of maternal cells crossing the placenta during MIA.

Methods and Results

Studies of placental cells often utilize BeWo cells (ATCC Cat# CCL-98), a choriocarcinoma cell line that forms a monolayer in culture and retains many of the hormone-secreting and polarized trans-celullar transport properties of human trophoblast cells (Pattillo and Gey, 1968; Liu et al., 1997). Because IL-6 increases endothelial permeability *in vivo* (Paul et al., 2003) and *in vitro* (Maruo et al., 1992), we hypothesized that BeWo cells may show increased permeability in response to IL-6 or poly(I:C). We

confirmed previous observations that BeWo cells respond to IL-6 (Stephanou and Handwerger, 1994) and express toll-like receptor TLR3. Western blots for phosphorylated STAT3 (pSTAT3) indicated that BeWo cells can respond to IL-6, with maximal STAT-3 activation occurring 15-30 min after exposure (data not shown). We also found that BeWo cells produce IL-6 in response to poly(I:C) (data not shown). Therefore, following the method of Liu et al. (1997), we assayed transport across a BeWo cell monolayers on transwell membranes. Cells were exposed to IL-6 (100 ng/ml) for 2 or 6 hours, and Fluorescein (MW 376) was added to the upper well of the plate. Permeability was calculated by measuring the amount of dye present in the lower well. Fluorescein permeability is not mediated by a specific transporter and therefore measures the integrity of the tight junctions formed by the cells. Whereas endothelial cells increase their permeability in response to IL-6 (Maruo et al., 1992), the placental cells did not. Preincubation with IL-6 caused the BeWo cells to decrease their permeability at 2 hrs (Fig. 1). Similar data were obtained with FD-04 (Sigma), a Fluorescein-conjugated dextran of MW 4000, but due to the lower permeability of the larger molecule, the signal-to-noise ratio was larger and the effect of IL-6 was not significant.

To verify the activation of STAT3 in the placenta *in vivo*, we assayed pSTAT3 in placental extracts by Western blotting following three hours following injection with poly(I:C), a time of maximal cytokine levels. The results indicate that the placenta does respond to poly(I:C) *in vivo* (Fig. 2). We next assessed the permeability of the placenta *in vivo*. Pregnant C57 females were injected with 20 mg/kg poly(I:C), and after 2.5 hrs were injected via the tail vein with 7.5 mg of fluorescently labeled FD04. After 10 min, the mice were sacrificed and maternal serum, placenta, embryos and amniotic fluid were

collected. Upon visual inspection, the bladder was very yellow, indicating that FD04 was filtered by the kidneys and concentrated in the bladder. High levels of FD04 were detectable in maternal serum, and low but detectable levels of FD04 were found in amniotic fluid and homogenates of fetuses and placentas. The level of FD04 in tissues was normalized to the level in maternal serum; differences in maternal serum concentration probably represent differences in quality of tail vein injection rather than differences in clearance related to poly(I:C) exposure because the tail vein injection of the poly(I:C) animal was poor, with about 75% of the FD-04 properly injected. There was significantly less FD04 in poly(I:C)-exposed placentas, perhaps indicating vascular constriction in the placenta (Fig. 2B) ($p < 0.005$). A lower level of FD04 was also seen in poly(I:C)-exposed embryos, which would be consistent with the BeWo experiments, but this difference was not significant ($p = 0.12$); however this could also simply reflect the lower placental levels. Interestingly, significantly higher levels of FD04 were found in the amniotic fluid of poly(I:C)-exposed embryos. These preliminary results require confirmation, possibly using radioactively labeled proteins to achieve greater sensitivity. However, this experiment did demonstrate that placental permeability to molecules in the small protein size range is intact in the poly(I:C)-exposed dam, suggesting that a gross, non-specific breakdown of placental integrity is an unlikely mechanism of poly(I:C) action. To investigate whether a poly(I:C)-driven increase in placental permeability could lead to fetal nutrient restriction, follow-up experiments could include examination of radiolabeled glucose transport.

The question of transport of maternal cells across the placenta was addressed by mating female mice heterozygous for an eGFP transgene, under control of the human

ubiquitin c promoter (UBC-GFP, Jackson Labs), with wild-type males. Half of the resulting embryos were therefore GFP^- embryos in a GFP^+ female, so that maternal-to-fetal transmission could be visualized. UBC-GFP mice were chosen because GFP expression is strong in leukocytes, and the intensity of GFP expression varies with cell type, so that different cell types can be identified by GFP intensity using flow cytometry (Schaefer et al., 2001). In retrospect, however, these mice were a poor choice because the GFP expression was not strong enough to be easily observed without anti-GFP antibody staining. A line with stronger GFP expression, such as that used by Zhou et al. (2000) should be used if this experiment is repeated.

Pregnant females were injected with 20 mg/kg poly(I:C) i.p. on E12.5 and sacrificed 12, 24 or 48 hours later. Embryos were serially sectioned sagitally at 20 μm. Due to low GFP expression, sections were stained with an anti-GFP antibody, Alexa488 secondary antibody and DAPI to visualize nuclei. Sections were observed at 20x magnification and maternal cells were counted when a GFP^+ cell with a $DAPI^+$ nucleus was found in the embryo. In a preliminary experiment, significantly more GFP^+ cells were found in poly(I:C)-exposed embryos (Fig. 3A). Two to three pregnant females were used for each time point; N of 20 μm sections and (embryos) = 33(6), 6(2), 28(6) for control, 12 hours and 24 hours. The GFP^+ cells are most commonly observed in large blood vessels, and occasionally seen in the heart, liver, or as shown in Fig. 3, the nasal epithelium. The GFP^+ cells in found in the $GFP^{-/-}$ embryos are primarily $Mac\text{-}1^+$ macrophages, however, rare $GFP^+/Mac1^-$ cells were also observed. The significance of this small number of cells in the embryo (90 to 150 maternal cells per embryo) is difficult to determine. Moreover, maternal lymphocytes are known to infiltrate the fetus during

normal pregnancy (Piotrowski and Croy, 1996; Petit et al., 1997; Lo et al., 1998), and preliminary observations from embryos collected 48 hours after poly(I:C) injection, on E14.5, revealed similar numbers of GFP^+ cells in control and poly(I:C)-exposed embryos (data not shown). Finally, maternal cells were never observed in the fetal brain, nor in the single adult offspring brain that was studied in a preliminary manner. It should also be noted that an attempt to replicate the early (E12.5) result failed; similar numbers of GFP^+ cells were found in control (8 cells in 40 sections) and poly(I:C) embryos (4 cells in 56 sections). Thus, while this is a potentially interesting observation, it is difficult to interpret the results.

The limiting factor in the cell transfer experiment is the time-consuming method of sectioning each embryo, scanning the sections at high magnification to look for cells, and eliminating (a large number of) false-positives by showing co-localization of DAPI and GFP. It may be possible to use automated cell sorting to detect, enrich and characterize the maternal cells found in the fetus; (Zhou et al (2000) used this method with lymphatic organs of E18 embryos. However, at E12.5 this would be more difficult. The limit of detection for the cell sorter is 1 in 10^5-10^6 cells (S. Diamond, personal communication). Thus, the detection of ~100 cells in an entire embryo would be nearing, if not exceeding, the limit of detection. Another possibility would be to use real-time quantitative PCR of fetal total DNA, looking for GFP, as a high-throughput method to quantify maternal-to-fetal transmission.

Perspectives

These preliminary experiments suggest that placental permeability and maternal-fetal cell transfer are not grossly altered by MIA. However, it is clear that IL-6 and poly(I:C) can activate STAT3 in placental cells *in vitro* and *in vivo*. Moreover, preliminary results suggest that placental permeability may also be altered *in vitro* and *in vivo*. Thus, these results may be worth following up.

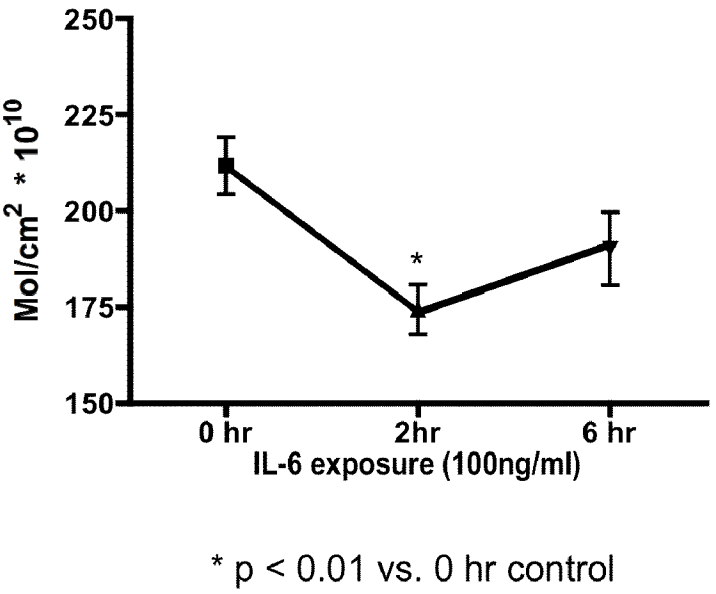
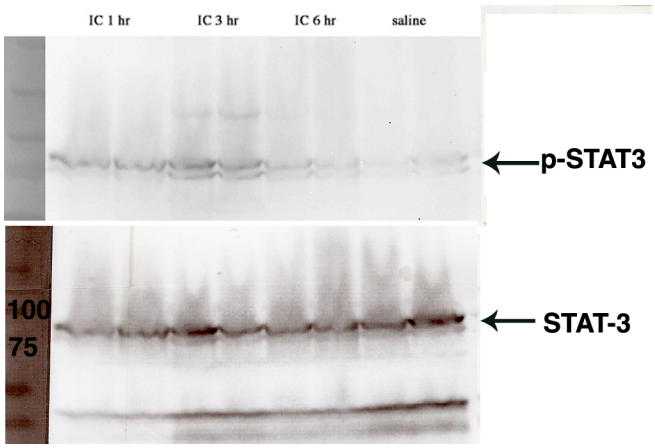


Figure 1: Transport of fluorescein across a BeWo cell monolayer is altered by IL-6. Confluent BeWo cells grown on transwell membranes were exposed to IL-6 (100 ng/ml) for 2 or 6 hours (total exposure time), and fluorescein (MW 376) was added to the upper well of the plate. After 2 hours, permeability was calculated by measuring the amount of dye present in the lower well. Each data point represents the average of three separate experiments, 3-5 wells per experiment. Similar data were obtained with FD-04, a fluorescein-conjugated dextran of MW 4000, but due to the lower permeability of the larger molecule, the signal-to-noise ratio was larger, and the differences were not significant.

A



B

	Control	Poly(I:C)
Embryos	0.28 ± 0.06 %	0.18 ± 0.03 %
Placentas	2.01 ± 0.08 %	1.67 ± 0.05 %*
Amniotic Fluid	0.15 ± 0.01 %	0.42 ± 0.01 %*

Fig. 2. The mouse placenta responds to poly(I:C) but does not alter its permeability to 4000 kD dextran. (A) Western blot analysis shows phosphorylated STAT-3 in the placenta of mice 1, 3 or 6 hrs after maternal injection of poly(I:C) (IC) or saline. P-STAT-3 is likely a marker of an IL-6 mediated response, but may reflect activity of other cytokines as well. Unphosphorylated STAT-3 is shown as a loading control. (B) The permeability of the placenta does not change three hours after maternal poly(I:C) injection. Fluorescent dextran (MW 4000) was injected into the female via the tail vein 30 minutes before sacrifice. Numbers indicates the percent of florescence measured in various homogenized tissues relative to fluorescence levels in the maternal serum. Numbers indicate mean ± SEM; N= one female per group; 6 control 10 poly(I:C) embryos. * $p < 0.005$

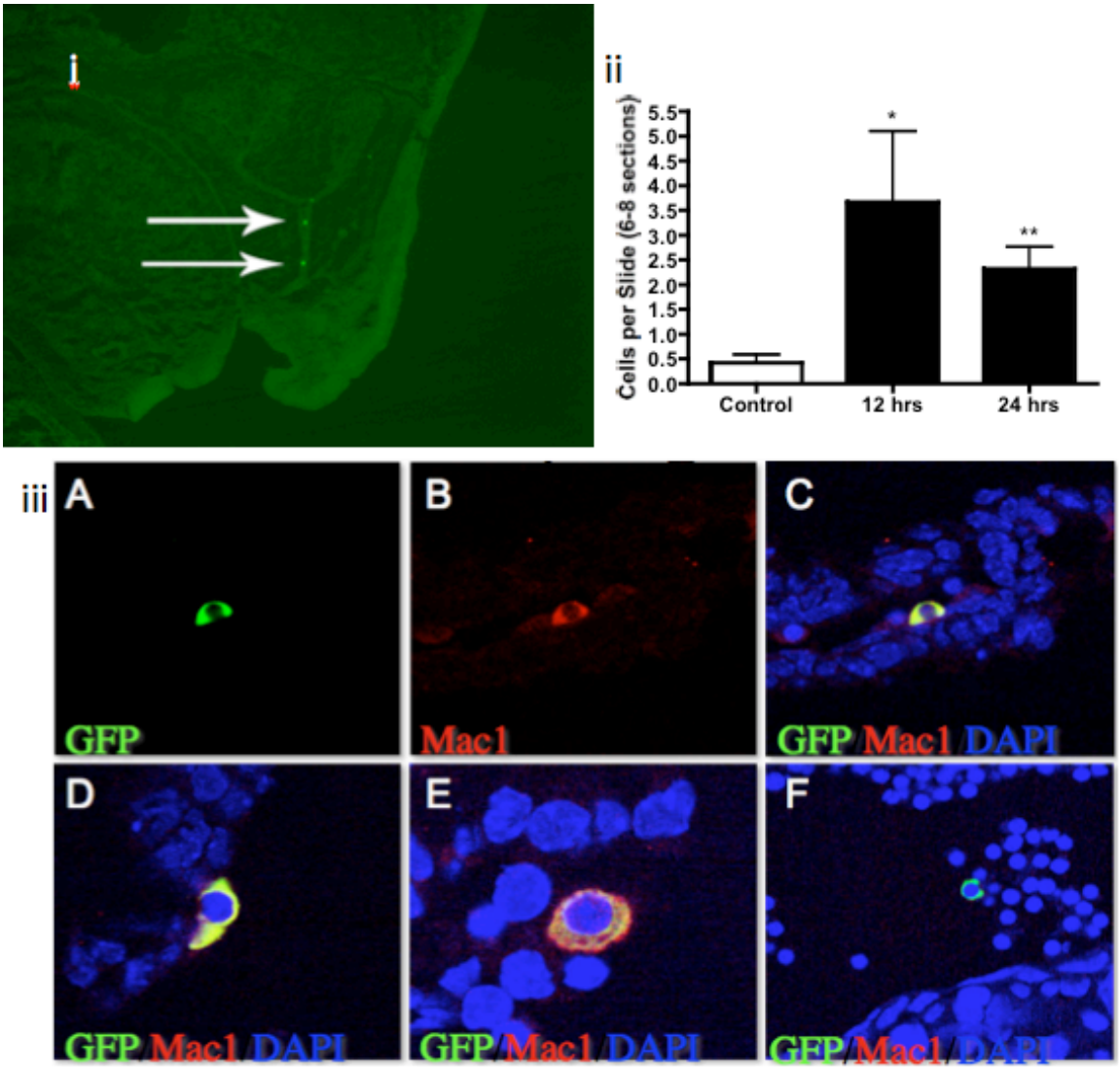


Fig3: GFP+ cells are observed in GFP-/- embryos that are growing in GFP +/- females. (i) Females were injected with 20 mg/kg poly(I:C) 12 or 24 hours before embryo collection. The GFP+ cells are most commonly observed in large blood vessels, and occasionally seen in the heart, liver, or as shown here, the nasal epithelium. (ii) In embryos collected 12 or 24 hours after poly(I:C) injection, more GFP^{+} cells are found compared to controls. Data are expressed as GFP^{+} cells per slide, each slide contains 6-8 embryo sections, depending on the size of the sections. (iii) The GFP^{+} cells found in the $GFP^{-/-}$ embryos are primarily macrophages, as assayed by Mac1 staining (A-F). Mac-

1^+/GFP^+ cells are observed adhered to the walls of blood vessels (D), or as a component of fetal blood (E). Rarely, a GFP^+/$Mac1^-$ cell is observed (F).

References

Artlett CM, Ramos R, Jiminez SA, Patterson K, Miller FW, Rider LG (2000) Chimeric cells of maternal origin in juvenile idiopathic inflammatory myopathies. Childhood Myositis Heterogeneity Collaborative Group. Lancet 356:2155-2156.

Arvola M, Gustafsson E, Svensson L, Jansson L, Holmdahl R, Heyman B, Okabe M, Mattsson R (2000) Immunoglobulin-secreting cells of maternal origin can be detected in B cell-deficient mice. Biol Reprod 63:1817-1824.

Ashwood P, Wills S, Van de Water J (2006) The immune response in autism: a new frontier for autism research. J Leukoc Biol 80:1-15.

Dahlgren J, Samuelsson AM, Jansson T, Holmang A (2006) Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr Res 60:147-151.

Debnath M, Chaudhuri TK (2006) The role of HLA-G in cytokine homeostasis during early pregnancy complicated with maternal infections: a novel etiopathological approach to the neurodevelopmental understanding of schizophrenia. Med Hypotheses 66:286-293.

Jones AL, Mowry BJ, Pender MP, Greer JM (2005) Immune dysregulation and self-reactivity in schizophrenia: do some cases of schizophrenia have an autoimmune basis? Immunol Cell Biol 83:9-17.

Khosrotehrani K, Guegan S, Fraitag S, Oster M, de Prost Y, Bodemer C, Aractingi S (2006) Presence of chimeric maternally derived keratinocytes in cutaneous

inflammatory diseases of children: the example of pityriasis lichenoides. J Invest Dermatol 126:345-348.

Liu F, Soares MJ, Audus KL (1997) Permeability properties of monolayers of the human trophoblast cell line BeWo. Am J Physiol 273:C1596-1604.

Lo ES, Lo YM, Hjelm NM, Thilaganathan B (1998) Transfer of nucleated maternal cells into fetal circulation during the second trimester of pregnancy. Br J Haematol 100:605-606.

Maruo N, Morita I, Shirao M, Murota S (1992) IL-6 increases endothelial permeability in vitro. Endocrinology 131:710-714.

Neugebauer R (2005) Accumulating evidence for prenatal nutritional origins of mental disorders. Jama 294:621-623.

Pardo CA, Eberhart CG (2007) The neurobiology of autism. Brain Pathol 17:434-447.

Pattillo RA, Gey GO (1968) The establishment of a cell line of human hormone-synthesizing trophoblastic cells in vitro. Cancer Res 28:1231-1236.

Paul R, Koedel U, Winkler F, Kieseier BC, Fontana A, Kopf M, Hartung HP, Pfister HW (2003) Lack of IL-6 augments inflammatory response but decreases vascular permeability in bacterial meningitis. Brain 126:1873-1882.

Petit T, Dommergues M, Socie G, Dumez Y, Gluckman E, Brison O (1997) Detection of maternal cells in human fetal blood during the third trimester of pregnancy using allele-specific PCR amplification. Br J Haematol 98:767-771.

Piotrowski P, Croy BA (1996) Maternal cells are widely distributed in murine fetuses in utero. Biol Reprod 54:1103-1110.

Sargent IL, Borzychowski AM, Redman CW (2006) NK cells and human pregnancy--an inflammatory view. Trends Immunol 27:399-404.

Schaefer BC, Schaefer ML, Kappler JW, Marrack P, Kedl RM (2001) Observation of antigen-dependent CD8+ T-cell/ dendritic cell interactions in vivo. Cell Immunol 214:110-122.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Stephanou A, Handwerger S (1994) Interleukin-6 stimulates placental lactogen expression by human trophoblast cells. Endocrinology 135:719-723.

Stevens AM (2007) Do maternal cells trigger or perpetuate autoimmune diseases in children? Pediatr Rheumatol Online J 5:9.

Stevens AM, Hermes HM, Rutledge JC, Buyon JP, Nelson JL (2003) Myocardial-tissue-specific phenotype of maternal microchimerism in neonatal lupus congenital heart block. Lancet 362:1617-1623.

Su EC, Johnson KL, Tighiouart H, Bianchi DW (2008) Murine Maternal Cell Microchimerism: Analysis Using Real-Time PCR and In Vivo Imaging. Biol Reprod:biolreprod.107.063305.

Susser E, Neugebauer R, Hoek HW, Brown AS, Lin S, Labovitz D, Gorman JM (1996) Schizophrenia after prenatal famine. Further evidence. Arch Gen Psychiatry 53:25-31.

Susser ES, Lin SP (1992) Schizophrenia after prenatal exposure to the Dutch Hunger Winter of 1944-1945. Arch Gen Psychiatry 49:983-988.

Torrey EF, Yolken RH (2001) The schizophrenia-rheumatoid arthritis connection: infectious, immune, or both? Brain Behav Immun 15:401-410.

van Os J (1997) Schizophrenia after prenatal famine. Arch Gen Psychiatry 54:577-578.

Wrenshall LE, Stevens ET, Smith DR, Miller JD (2007) Maternal microchimerism leads to the presence of interleukin-2 in interleukin-2 knock out mice: implications for the role of interleukin-2 in thymic function. Cell Immunol 245:80-90.

Zaretsky MV, Alexander JM, Byrd W, Bawdon RE (2004) Transfer of inflammatory cytokines across the placenta. Obstet Gynecol 103:546-550.

Zhou L, Yoshimura Y, Huang Y, Suzuki R, Yokoyama M, Okabe M, Shimamura M (2000) Two independent pathways of maternal cell transmission to offspring: through placenta during pregnancy and by breast-feeding after birth. Immunology 101:570-580.

Appendix C

Gene-Environmental Interactions in Mental Disease:

Maternal Immune Activation in a DISC-1 Mutant Mouse

Stephen Smith and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

Rationale

Schizophrenia and autism both have a strong genetic component, as the concordance rate of monozygotic twins is about 50% for schizophrenia, and between 60-90% in autism (Lemery-Chalfant et al., 2006). However, those rates also suggest that genes are not sufficient to cause mental disease, and it is clear that environmental factors such as maternal infection (Brown and Susser, 2002; Patterson, 2002; Fatemi, 2005) are also important risk factors. In fact, most current theories of mental disease posit that the interaction of genetic and environmental factors contributes to the overall risk of developing mental disease (for review, see (Tsuang et al., 2004)). For example, perhaps a genetically encoded hyper-responsiveness to a component of the maternal immune response causes some offspring of mothers who develop infections during pregnancy to go on to develop mental diseases, while others do not. Several mouse strains have been developed recently that express genes that have been identified as risk factors for schizophrenia and autism. These mice can now be tested in the maternal immune activation (MIA) paradigm, which has face and construct validity, allowing us to directly test the gene-environment interaction theory *in vivo*.

The disrupted-in-schizophrenia-1 (DISC-1) gene was first identified in a large Scottish family with a history of violence and psychiatric disorders, including schizophrenia (Millar et al., 2000). The DISC-1 gene was identified at the break-point of a chromosomal translocation that segregated with mental disease in this family. Subsequent linkage and association studies also link DISC-1 mutations with schizophrenia (Hennah et al., 2003; Hennah et al., 2007; Hennah et al., 2008). DISC-1 has also been associated with autism in one study (Kilpinen et al., 2008). The DISC-1

protein associates with NUDEL and LIS1, and likely forms a component of the microtubule-associated dynein motor complex, which is involved in neuronal migration (Ozeki et al., 2003; Brandon et al., 2004). In fact, DISC-1 promotes neurite outgrowth *in vitro* (Miyoshi et al., 2003) and migration of cortical neurons *in vivo* (Kamiya et al., 2005). Importantly, the truncated form of DISC-1 associated with psychiatric disease lacks these binding and functional properties (Miyoshi et al., 2003; Brandon et al., 2004; Kamiya et al., 2005).

The commonly used mouse strain, 129Sv, was found to have a 25 kb deletion in the endogenous DISC-1 gene, which introduced a premature stop codon and resulted in a truncated protein similar to the predicted truncated human form (Clapcote and Roder, 2006; Koike et al., 2006). The Gogos group introduced a second stop codon to ensure truncation of the protein, and back-crossed the mouse onto a C57/bl background, a strain that has a normal, functional DISC-1 gene. The DISC-$1^{mut/mut}$ mice display impaired working memory compared to control littermates (Koike et al., 2006), which makes the mice one of the first available models in which a presumed loss of function genetic change linked to a subset of schizophrenia cases can also cause behavioral abnormalaites in a mouse. We obtained these DISC-1 mice from the Gogos lab to test whether environmental and genetic risk factors for schizophrenia can interact to produce more severe behavioral deficits than either risk factor alone. Furthermore, the DISC-1 model may be an excellent candidate to interact with the maternal immune activation (MIA) model because both DISC-1 (Kamiya et al., 2005) and MIA (Limin Shi, personal communication) can alter neuronal migration in the fetal brain. Thus, both risk factors could be acting in a common pathological pathway.

Preliminary Work

DISC-1$^{+/-}$ males and females were mated in the Caltech animal facility, and pregnant females were injected with 20 mg/kg poly(I:C) I.P. on E12.5 of pregnancy. The offspring were raised by the mother, weaned at three weeks, and behaviorally tested as previously described (Smith 2007). After behavioral testing was completed, the mice were genotyped and behavioral data was analyzed by 2-way ANOVA, with treatment (poly(I:C) vs. control) and genotype the main variables.

Before genotyping, alterations in several behaviors affirmed that poly(I:C) treatment causes behavioral deficits in the genetic background of DISC-1 mice (Fig. 1). Although there are no significant differences in prepulse inhibition (PPI), there is a significant difference between poly(I:C) and control animals in freezing in the latent inhibition (LI) test. Strangely, the control and NPE groups are not significantly different, so neither group shows LI as we have previously defined it. However, this is likely due to the small number of animals and lower-than-normal freezing rate in the NPE group, which could possibly be corrected by the addition of a few more animals to the groups. The fact that the control and poly(I:C) groups are significantly different indicate that poly(I:C) had the expected effect. The MIA offspring also show significantly fewer entries into the center of the open field and less distance moved, consistent with previous behavior results (Smith 2007, Shi 2003). However, in the social interaction test, the MIA offspring do not show significant differences.

After genotyping, the results suggest that the effect of poly(I:C) does not interact with the genotype of the offspring (Fig. 2). Genotype does not affect behavior of the

offspring in PPI, LI or open field behavior. Genotype has a significant effect in the social interaction test, however, with DISC1$^{-/-}$ mice showing more of a preference for social interaction than their wildtype littermates. This unexpected result could explain the lack of social interaction deficits in the overall MIA offspring. Finally, 2-way ANOVA reveals a significant treatment x genotype interaction for center entries, indicating that control DISC-1 mutant mice show less anxiety in the open field than wild-type littermates, while poly(I:C)-treated DISC-1 mutant mice show more anxiety.

Future Directions

These preliminary results suggest that the 129SvDISC-1 mutation does not interact with MIA to produce a more severe, schizophrenia-like phenotype in the offspring. However, this result may reflect limitations in mouse model that we chose to use, and may not translate to other DISC-1 models. Recently, several other DISC-1 models have been characterized (see Table 1). These mice all show abnormal behaviors that are relevant to schizophrenia, such as PPI deficits and hyperactivity. Interestingly, the specific nature of the DISC-1 mutation is very important, as highlighted by Clapcote et al. (2007), who showed that two different ENU-induced point mutations in DISC-1 resulted in distinct behavioral phenotypes, one schizophrenia-like and one depression-like. All of the more recently engineered DISC-1 models tend to have more extensive behavioral and histological abnormalities than the Gogos model that we used. It is possible that the 129SvDISC-1 is still partially active, or there is some read-through of the stop codons. A recent paper showed that several isoforms of DISC-1 are, in fact, detectable in the 129SvDISC-1 model by Western blotting, despite the fact that two

premature stop codons should have prevented expression (Ishizuka et al., 2007). Repeating the experiment with a different DISC-1 strain may yield different results.

In fact, at least two other groups are actively working on this same experiment, using the poly(I:C) model and different DISC-1 models. Preliminary results from a group using the strain of Hikida et al. (2007) suggested an interaction between poly(I:C) and the CamKII-DN-DISC-1 genotype, but the results were not particularly impressive (Nikolskaia et al., 2007). In addition, the group using the strains reported by Clapcote et al. (2007) showed significant interaction between poly(I:C) administration and genotype,with severe embryo loss caused by poly(I:C) in the L100P (schizophrenia-like) mouse, but not in the Q31L (depression-like) mouse (Lipina et al., 2007). This result highlights the importance of the specific type of DISC-1 mutation, and may explain why the Gogos strain that we have used does not appear to show an MIA-genotype interaction while the other DISC-1 models do. We are collaborating with the Lipina group to further characterize the behavioral and histological phenotypes of the MIA-DISC-1 interactions in these strains.

Reference	Method	Behavior	Histology
(Koike et al., 2006)	Natural 25bp deletion in 129 mice backcrossed onto a C57 background	Working memory deficit	Grossly normal
(Li et al., 2007)	TM-inducible fragment of DISC-1 expressed under CamKII promoter acts as dominant-negative; TM PND7	Working memory deficit, increased immobility in forced swim, social interaction time decreased	Reduced dendritic branching in the dentate gyrus; reduced synaptic transmission in hippocampus*
(Hikida et al., 2007)	A fragment of DISC-1 expressed under CamKII promoter acts as dominant-negative	Hyperactivity in open field; PPI deficit at 1 of 5 pre-pulse intensities; increased immobility in forced swim, normal social interaction, water maze, working	Reduced parvalbumin staining in 1 of 2 lines,

		memory	
(Clapcote et al., 2007)	ENU-mutagenesis produced two exon2 point mutations; Q31L and L100P	**Q31L**: PPI, LI, working memory, impairments; decreased social interaction, sucrose drinking; increased immobility in forced swim **L100P**: PPI, LI, working memory impairments, open field hyperactivity,	Reduced brain volume in both strains

Table 1: Summary of DISC-1 mutant mouse strains. Behavioral and histological data is shown for the five published studies of DISC-1 mutant mice. Abbreviations: bp base pairs; ENU N-nitroso-N-ethylurea; LI latent inhibition; PND post-natal day (age); PPI prepulse inhibition; Tet tetracycline; TM Tamoxifin; TRE tetracycline-responsive element. * indicates electrophysiology result.

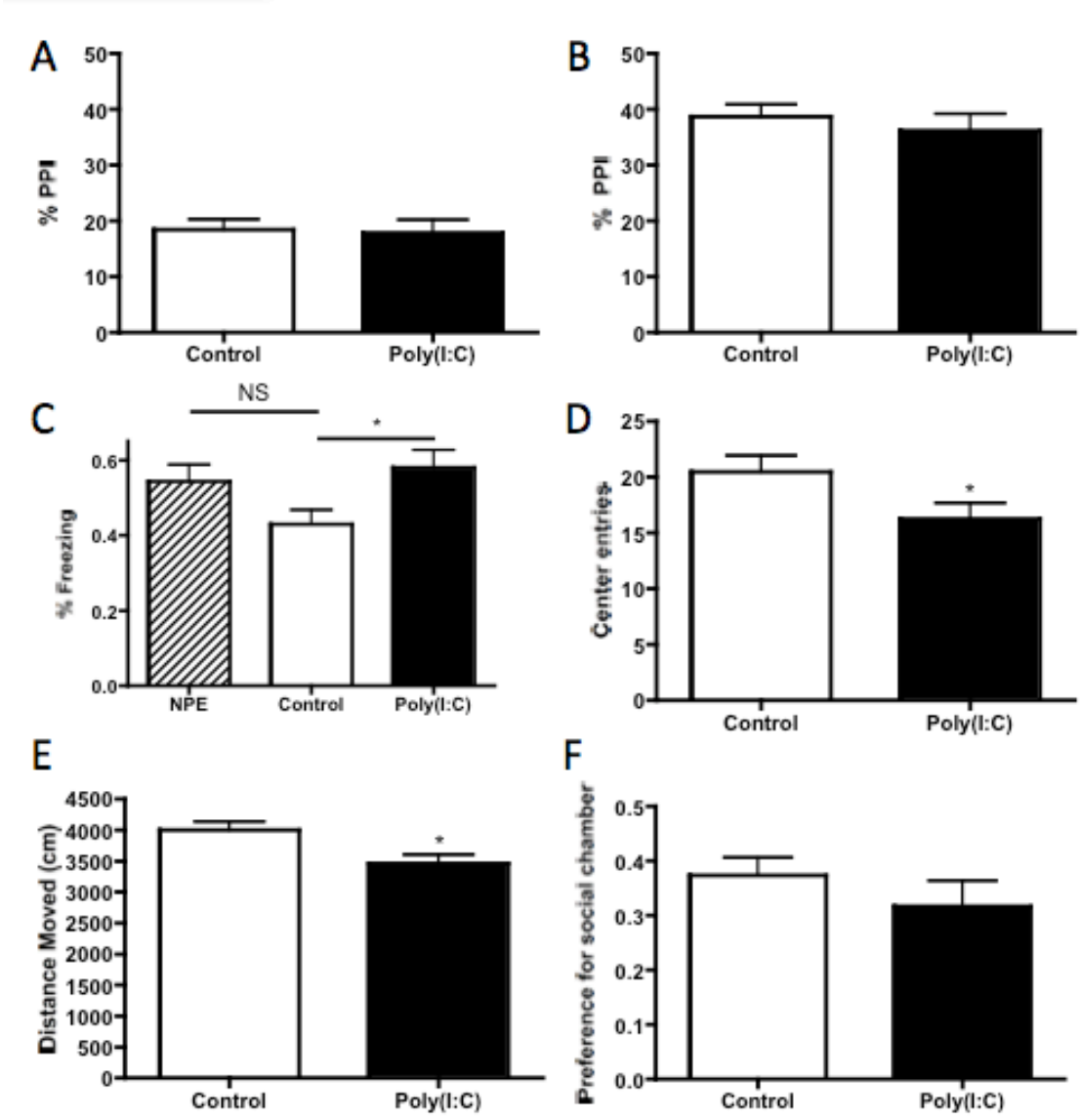


Figure 1. Behavioral results before genotyping. Before genotyping the offspring, alterations in several behaviors affirm that maternal poly(I:C) treatment causes behavioral deficits in the 129SvDISC-1 mice. Although there are no significant differences in PPI (A,B) there is a significant difference in freezing in the latent inhibition test (C) and significantly fewer entries into the center and less distance moved in the open field (D,E). In the social interaction test (F), MIA offspring do not show significant differences from control offspring.

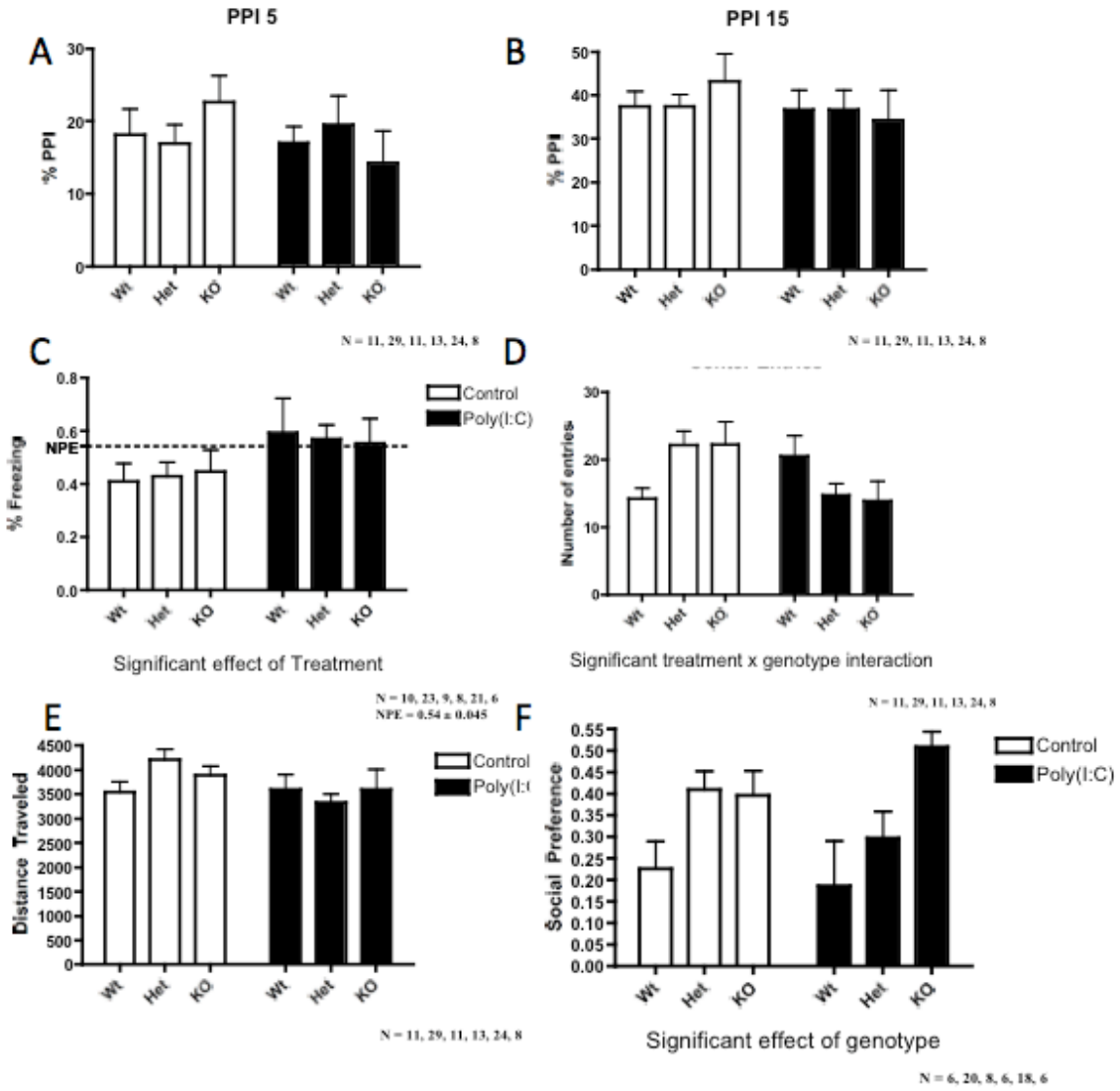


Figure 2. Behavioral results after genotyping. The effect of maternal poly(I:C) does not interact with the DISC-1 genotype of the offspring. Genotype does not effect PPI (A,B) LI (C), or open field behavior (D, E). Genotype has a significant effect in the social interaction test, with DISC1$^{-/-}$ mice unexpectedly being more social than wildtype littermates (F). 2-way ANOVA reveals a significant treatment x genotype interaction for center entries (D), but the nature of this interaction is not clear.

References

Brandon NJ, Handford EJ, Schurov I, Rain JC, Pelling M, Duran-Jimeniz B, Camargo LM, Oliver KR, Beher D, Shearman MS, Whiting PJ (2004) Disrupted in Schizophrenia 1 and Nudel form a neurodevelopmentally regulated protein complex: implications for schizophrenia and other major neurological disorders. Mol Cell Neurosci 25:42-55.

Brown AS, Susser ES (2002) In utero infection and adult schizophrenia. Ment Retard Dev Disabil Res Rev 8:51-57.

Clapcote SJ, Roder JC (2006) Deletion polymorphism of Disc1 is common to all 129 mouse substrains: implications for gene-targeting studies of brain function. Genetics 173:2407-2410.

Clapcote SJ, Lipina TV, Millar JK, Mackie S, Christie S, Ogawa F, Lerch JP, Trimble K, Uchiyama M, Sakuraba Y, Kaneda H, Shiroishi T, Houslay MD, Henkelman RM, Sled JG, Gondo Y, Porteous DJ, Roder JC (2007) Behavioral phenotypes of Disc1 missense mutations in mice. Neuron 54:387-402.

Fatemi SH (2005) Prenatal human influenza viral infection, brain development and schizophrenia. in Neuropsychiatric disorders and infeciton (ed S H Fatemi) pp 66-83 Taylor and Francis, UK.

Hennah W, Tomppo L, Hiekkalinna T, Palo OM, Kilpinen H, Ekelund J, Tuulio-Henriksson A, Silander K, Partonen T, Paunio T, Terwilliger JD, Lonnqvist J, Peltonen L (2007) Families with the risk allele of DISC1 reveal a link between

schizophrenia and another component of the same molecular pathway, NDE1. Hum Mol Genet 16:453-462.

Hennah W, Varilo T, Kestila M, Paunio T, Arajarvi R, Haukka J, Parker A, Martin R, Levitzky S, Partonen T, Meyer J, Lonnqvist J, Peltonen L, Ekelund J (2003) Haplotype transmission analysis provides evidence of association for DISC1 to schizophrenia and suggests sex-dependent effects. Hum Mol Genet 12:3151-3159.

Hennah W, Thomson P, McQuillin A, Bass N, Loukola A, Anjorin A, Blackwood D, Curtis D, Deary IJ, Harris SE, Isometsa ET, Lawrence J, Lonnqvist J, Muir W, Palotie A, Partonen T, Paunio T, Pylkko E, Robinson M, Soronen P, Suominen K, Suvisaari J, Thirumalai S, Clair DS, Gurling H, Peltonen L, Porteous D (2008) DISC1 association, heterogeneity and interplay in schizophrenia and bipolar disorder. Mol Psychiatry.

Hikida T, Jaaro-Peled H, Seshadri S, Oishi K, Hookway C, Kong S, Wu D, Xue R, Andrade M, Tankou S, Mori S, Gallagher M, Ishizuka K, Pletnikov M, Kida S, Sawa A (2007) Dominant-negative DISC1 transgenic mice displayschizophrenia-associated phenotypes detected by measures translatable to humans. Proc Natl Acad Sci U S A 104:14501-14506.

Ishizuka K, Chen J, Taya S, Li W, Millar JK, Xu Y, Clapcote SJ, Hookway C, Morita M, Kamiya A, Tomoda T, Lipska BK, Roder JC, Pletnikov M, Porteous D, Silva AJ, Cannon TD, Kaibuchi K, Brandon NJ, Weinberger DR, Sawa A (2007) Evidence that many of the DISC1 isoforms in C57BL/6J mice are also expressed in 129S6/SvEv mice. Mol Psychiatry 12:897-899.

Kamiya A, Kubo K, Tomoda T, Takaki M, Youn R, Ozeki Y, Sawamura N, Park U, Kudo C, Okawa M, Ross CA, Hatten ME, Nakajima K, Sawa A (2005) A schizophrenia-associated mutation of DISC1 perturbs cerebral cortex development. Nat Cell Biol 7:1167-1178.

Kilpinen H, Ylisaukko-Oja T, Hennah W, Palo OM, Varilo T, Vanhala R, Nieminen-von Wendt T, von Wendt L, Paunio T, Peltonen L (2008) Association of DISC1 with autism and Asperger syndrome. Mol Psychiatry 13:187-196.

Koike H, Arguello PA, Kvajo M, Karayiorgou M, Gogos JA (2006) Disc1 is mutated in the 129S6/SvEv strain and modulates working memory in mice. Proc Natl Acad Sci U S A 103:3693-3697.

Lemery-Chalfant K, Goldsmith HH, Schmidt NL, Arneson CL, Van Hulle CA (2006) Wisconsin Twin Panel: current directions and findings. Twin Res Hum Genet 9:1030-1037.

Li W, Zhou Y, Jentsch JD, Brown RA, Tian X, Ehninger D, Hennah W, Peltonen L, Lonnqvist J, Huttunen MO, Kaprio J, Trachtenberg JT, Silva AJ, Cannon TD (2007) Specific developmental disruption of disrupted-in-schizophrenia-1 function results in schizophrenia-related phenotypes in mice. Proc Natl Acad Sci U S A 104:18280-18285.

Lipina TV, Zai C, Hlousek D, Roder JC, Wong AHC (2007) Effects of maternal immune activation in utero on behavior of DISC1 mutant mouse offspring: implications for the gene-environment interaction model in schizophrenia. 2007 Society for Neuroscience poster presentation.

Millar JK, Wilson-Annan JC, Anderson S, Christie S, Taylor MS, Semple CA, Devon RS, Clair DM, Muir WJ, Blackwood DH, Porteous DJ (2000) Disruption of two novel genes by a translocation co-segregating with schizophrenia. Hum Mol Genet 9:1415-1423.

Miyoshi K, Honda A, Baba K, Taniguchi M, Oono K, Fujita T, Kuroda S, Katayama T, Tohyama M (2003) Disrupted-In-Schizophrenia 1, a candidate gene for schizophrenia, participates in neurite outgrowth. Mol Psychiatry 8:685-694.

Nikolskaia O, Ayhan Y, Ross CA, Pletnikov M (2007) Cell and mouse models of gene-environment interactions in the pathogenesis of psychiatric conditions. 2007 Society for Neuroscience poster presentation.

Ozeki Y, Tomoda T, Kleiderlein J, Kamiya A, Bord L, Fujii K, Okawa M, Yamada N, Hatten ME, Snyder SH, Ross CA, Sawa A (2003) Disrupted-in-Schizophrenia-1 (DISC-1): mutant truncation prevents binding to NudE-like (NUDEL) and inhibits neurite outgrowth. Proc Natl Acad Sci U S A 100:289-294.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Tsuang MT, Bar JL, Stone WS, Faraone SV (2004) Gene-environment interactions in mental disorders. World Psychiatry 3:73-83.

Appendix D

Modeling Maternal Immune Activation in Rhesus Macaques: Measuring the Cytokine Response

Stephen Smith[1], Melissa Baumann[2], David Amaral[2] and Paul H. Patterson[1]

[1] Division of Biology, California Institute of Technology, Pasadena, CA

[2] Department of Psychiatry & Behavioral Sciences, The M.I.N.D. Institute, University of California, Davis, CA

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Rationale

Mice are a useful experimental animal in which to model the effects of maternal immune activation (MIA) due to their relatively fast generation times, the powerful genetic tools available, and the similarity between human and rodent brain development and anatomy. However, in modeling psychiatric diseases, a primate model may be able to better capture some of the essential components of the diseases, such as maternal-offspring interaction and social behavior (Bauman et al., 2004a, b; Martin et al., 2008). We have begun a collaboration with Dr. David Amaral, the California National Primate Research Center (CNPRC) and the M.I.N.D. Institute at UC Davis. In late 2007, the staff at the CNPRC began the process of selecting female rhesus macaques for mating, administering MIA to pregnant females, and tracking the development of the fetuses with ultrasound. M. Bauman and colleagues will closely monitor the offspring for a variety of social behaviors using a sophisticated ethogram developed by the Amaral group. The goal is to establish a MIA model in non-human primates so that human-like behavior can be quantified and related to similar behaviors seen in autism.

Injection of poly(I:C) into primates is complicated by the fact that, unlike rodents, primates have endogenous ribonucleases (RNAses) in their blood that quickly break down poly(I:C) and prevent it from inducing an innate immune response (Nordlund et al., 1970; de Clercq, 1979). However, complexing poly(I:C) with polylysine and carboxymethylcellulose (polyICLC) prevents RNAse-mediated degradation by an unknown mechanism (Levy et al., 1975; Sammons et al., 1977). This polyICLC induces strong activation of the innate immune system in humans and non-human primates, and is currently in phase-II clinical trials for treating malignant gliomas (Salazar et al., 1996),

and has been tested for treatment of human renal carcinoma and advanced lymphoma (Giantonio et al., 2001). We obtained polyICLC from Oncovir, the company that is performing the human trials, and administered the compound in various doses to pregnant monkeys. We are currently characterizing the maternal immune response to polyICLC, and tracking fetal development.

Preliminary work

We chose to administer polyICLC on days 43, 44, 46, 47, 49 and 50 (pregnancy is 145 days) for several reasons: First, we planned to use only male embryos, since autism is found in males four times as often as in females. For technical reasons, day 43 is the earliest possible time that we are able to determine the sex of the embryo. Moreover, this time period roughly corresponds to the rodent brain developmental milestones that occur when we inject mice with poly(I:C) (E12.5) (Clancy et al., 2001). Finally, based on human thalidomide and valproic acid studies (Rodier et al., 1996), we suspect that this time period may constitute the window of susceptibility for autism.

For the pilot study, doses of 0.5, 1, and 2 mg/kg were chosen based upon limited published data documenting the effect of low doses of polyICLC in primates (Puri et al., 1996) and on doses in rodent studies (4-20 mg/kg (Zuckerman et al., 2003; Ozawa et al., 2006; Meyer et al., 2007; Smith et al., 2007)). We administered polyICLC over seven days to mimic a week-long respiratory infection. The initial monkeys were given 0.5 mg/kg and when that dose was tolerated, some monkeys were given 1 mg/kg. Both doses caused significant sickness behavior for the seven-day course of treatment, and two pregnancies have been lost out of the 7 subjects receiving polyICLC thus far. We

therefore eliminated the projected 2 mg/kg dose from the protocol. Serum samples were collected at several time points during treatment: while the monkey was anesthetized for an ultrasound two days before and two days after treatment started, and three hours after polyICLC injection on days 1 and 7 of treatment. We only collected serum samples four times to minimize stress to the monkeys, and we collected on days 1 and 7 to determine if there was any desensitization to the polyICLC. Assays for serum IL-6 are being conducted at Caltech by ELISA according to the manufacturer's instructions (Cell Sciences, Canton, MA). Data indicate that polyICLC induces a strong innate immune response in monkeys injected with both 0.5 and 1 mg/kg (Table 1). It also appears that we may have hit a dosage ceiling, as the two doses produce similar levels of IL-6. In addition, IL-6 levels are similar to those found after poly(I:C) injection into pregnant mice (Smith et al. 2007), suggesting that these polyICLC doses should be sufficient to produce behavior deficits in the offspring, assuming the mechanism is similar to that in the mouse model. Moreover, as mentioned above, two pregnancies have been lost, which also occasionally happens at relatively high doses in the rodent model, again suggesting that we are at the appropriate dosage level.

Future Directions

The plan for this initial pilot study is to inject at least four pregnant monkeys per polyICLC dose as well as inject 3 monkeys with PBS in the control group. The behavioral analysis of the offspring (Bauman et al (2004a,b); Martin et al. (2008)) will begin soon after birth and continue for approximately a year. Based on the results from the pilot study, a larger cohort will be treated in the next breeding season beginning in

late 2008, and those offspring will be followed for at least 2 years. Analysis of the brains of these offspring will eventually be conducted by MRI and histology.

Monkey	Treatment	Baseline	First Injection	Final Injection	After Treatement
32892	*Control*	36	55	39	35
32772	*Control*	0	0	0	0
29522	*0.5 mg/kg*	0	42788	2338	57
32305	*0.5 mg/kg*	0	14303	3789	25
35106	*1 mg/kg*	0	23441	1200	0
33366	*1 mg/kg*	0	29080	1080	0

Table 1. PolyICLC induces interleukin-6 in pregnant macaque monkeys. Serum samples were assayed before treatment, three hours after the initial polyICLC injection, three hours after the final poly ICLC injection, and two days after treatment concluded. Values are expressed as pg/ml.

References:

Bauman MD, Lavenex P, Mason WA, Capitanio JP, Amaral DG (2004a) The development of social behavior following neonatal amygdala lesions in rhesus monkeys. J Cogn Neurosci 16:1388-1411.

Bauman MD, Lavenex P, Mason WA, Capitanio JP, Amaral DG (2004b) The development of mother-infant interactions after neonatal amygdala lesions in rhesus monkeys. J Neurosci 24:711-721.

Clancy B, Darlington RB, Finlay BL (2001) Translating developmental time across mammalian species. Neuroscience 105:7-17.

de Clercq E (1979) Degradation of poly(inosinic acid) - poly(cytidylic acid) [(I)n - (C)n] by human plasma. Eur J Biochem 93:165-172.

Giantonio BJ, Hochster H, Blum R, Wiernik PH, Hudes GR, Kirkwood J, Trump D, Oken MM (2001) Toxicity and response evaluation of the interferon inducer poly ICLC administered at low dose in advanced renal carcinoma and relapsed or refractory lymphoma: a report of two clinical trials of the Eastern Cooperative Oncology Group. Invest New Drugs 19:89-92.

Levy HB, Baer G, Baron S, Buckler CE, Gibbs CJ, Iadarola MJ, London WT, Rice J (1975) A modified polyriboinosinic-polyribocytidylic acid complex that induces interferon in primates. J Infect Dis 132:434-439.

Martin LA, Ashwood P, Braunschweig D, Cabanlit M, Van de Water J, Amaral DG (2008) Stereotypies and hyperactivity in rhesus monkeys exposed to IgG from mothers of children with autism. Brain Behav Immun.

Meyer U, Murray PJ, Urwyler A, Yee BK, Schedlowski M, Feldon J (2007) Adult behavioral and pharmacological dysfunctions following disruption of the fetal brain balance between pro-inflammatory and IL-10-mediated anti-inflammatory signaling. Mol Psychiatry.

Nordlund JJ, Wolff SM, Levy HB (1970) Inhibition of biologic activity of poly I: poly C by human plasma. Proc Soc Exp Biol Med 133:439-444.

Ozawa K, Hashimoto K, Kishimoto T, Shimizu E, Ishikura H, Iyo M (2006) Immune activation during pregnancy in mice leads to dopaminergic hyperfunction and cognitive impairment in the offspring: a neurodevelopmental animal model of schizophrenia. Biol Psychiatry 59:546-554.

Puri SK, Dutta GP, Levy HB, Maheshwari RK (1996) Poly ICLC inhibits Plasmodium cynomolgi B malaria infection in rhesus monkeys. J Interferon Cytokine Res 16:49-52.

Rodier PM, Ingram JL, Tisdale B, Nelson S, Romano J (1996) Embryological origin for autism: developmental anomalies of the cranial nerve motor nuclei. J Comp Neurol 370:247-261.

Salazar AM, Levy HB, Ondra S, Kende M, Scherokman B, Brown D, Mena H, Martin N, Schwab K, Donovan D, Dougherty D, Pulliam M, Ippolito M, Graves M, Brown

H, Ommaya A (1996) Long-term treatment of malignant gliomas with intramuscularly administered polyinosinic-polycytidylic acid stabilized with polylysine and carboxymethylcellulose: an open pilot study. Neurosurgery 38:1096-1103; discussion 1103-1094.

Sammons ML, Stephen EL, Levy HB, Baron S, Hilmas DE (1977) Interferon induction in cynomolgus and rhesus monkey after repeated doses of a modified polyriboinosinic-polyribocytidylic acid complex. Antimicrob Agents Chemother 11:80-83.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Zuckerman L, Rehavi M, Nachman R, Weiner I (2003) Immune activation during pregnancy in rats leads to a postpubertal emergence of disrupted latent inhibition, dopaminergic hyperfunction, and altered limbic morphology in the offspring: a novel neurodevelopmental model of schizophrenia. Neuropsychopharmacology 28:1778-1789.

Appendix E

Markers of Interleukin-6 Activation in the Fetal Brain

Stephen Smith, Elaine Hsiao, Ilana Goldflam and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

Rationale

Interleukin-6 (IL-6) was identified as a critical mediator in causing the changes in behavior and gene expression in the adult offspring of poly(I:C)-treated dams (Smith et al., 2007). Co-injection of poly(I:C) and anti-IL-6 antibodies prevents behavior deficits in the offspring, and IL-6 knockout mice are insensitive to the effects of the double-stranded RNA. IL-6 crosses both human (Zaretsky et al., 2004) and rat placentas (Dahlgren et al., 2006), which led us to hypothesize that in the maternal immune activation (MIA) poly(I:C) model, IL-6 activates signaling pathways in the fetal brain that alter fetal brain development and cause the observed abnormalities.

Interleukin-6 is a member of the IL-6 family of cytokines, which includes several other neuropoietic cytokines such as leukemia inhibitory factor (LIF), oncostatin M, interleukin-11, interleukin-27 and ciliary neurotrophic factor. The neuropoietic cytokines utilize the signal transducing co-receptor gp130, and each cytokine employs a specific co-receptor (Bauer et al., 2007). In the case of IL-6, its co-receptor (IL-6R) is found in membrane-bound and soluble forms, both of which are capable of transducing a signal when complexed with GP130. Many cells, including neurons in the fetal brain, express IL-6R (Gadient and Otten, 1994; Bauer et al., 2007). However, almost all cells in the body express GP130, and because of the activity of the soluble IL-6R, these cells are all potentially able to respond to IL-6 (Ringheim et al., 1998; McLoughlin et al., 2005). Thus, restricting the search for IL-6 signaling activation to cells that express the IL-6R may be problematic.

The IL-6R/GP130 complex activates Janus kinases (JAKs) and signal transducers

and activators of transcription (STATs), with the major step in the IL-6 signaling pathway being the phosphorylation and activation of STAT-3 (Bauer et al., 2007). Phosphorylated STAT-3 (pSTAT-3) then translocates to the nucleus and induces transcription of many genes, including cytokines, suppressor of cytokine signaling (SOCS) proteins, cytokine-induced Src homology 2 (SH2) protein (CIS), tissue inhibitor of metalloproteinase 1 (TIMP-1) and Pim-1. Thus, the search for the site of IL-6 action in the fetal brain has at least three major possible targets: IL-6 protein itself, components of the IL-6 signal transduction pathway (i.e. pSTAT-3) and genes induced by IL-6.

Preliminary Work

First, the fetal brain was stained for IL-6R expression to see if the fetal brain had the potential to respond to IL-6. E12 embryos were collected from anesthetized females, fixed for four hours in 0.4% PFA in PBS, rinsed in PBS several times, put through a sucrose gradient (10%, 20%, 30%) and frozen at -80 C. Embryos were then sectioned and stained with an anti-mouse IL-6R antibody (R&D Systems). Using immunofluorescent (Alexa488-conjugated) secondary antibody, no staining was observed above the low background level seen in the no-primary-antibody control. However, using a biotinylated secondary antibody for signal amplification and immunoperoxidase staining, IL-6R-immunoreactivity was observed as punctate staining on the membranes of cells in fetal brain (Fig. 1) that were not visible in no-primary-antibody control sections. This agrees with published reports that IL-6R is present in mouse and human fetal brain (Ulfig and Friese, 1999; Dame and Juul, 2000).

Several groups have reported increased IL-6 protein or mRNA in the fetal brain following MIA in rodents (see Chapter 2, Table II). We injected 20 mg/kg poly(I:C) i.p. into pregnant C57 females on E12.5 to induce MIA, and collected embryos 3 hours later. Heads were removed and frozen at -80. Heads were subsequently weighed, and while still frozen immersed in 50 µl PBS + 0.05% Tween-20 with protease inhibitors (Roche) and briefly sonicated to disrupt cellular membranes. All of the resulting extract was placed in a single well of an ELISA assay (R&D Systems) and the reaction run according to a modified protocol (see the ELISA protocol, below). Levels of IL-6 in the embryonic heads were very low, and many of the samples were below the limits of detection for the assay. However, we did detect higher average levels of IL-6 in the heads of poly(I:C)-injected embryos (Fig. 2a). Due to the small number of embryos (N = 8 control and 4 poly(I:C)), differences were not significant ($p = 0.15$). Although this experiment needs to be repeated with a higher N, a positive result would agree with published data showing IL-6 increases in fetal brain after MIA.

Next, we attempted to visualize cells that are responding to IL-6 in the fetal brain directly, using immunoflourescent staining for p-STAT-3. We collected embryos from poly(I:C)-treated and control females and sections were stained with a p-STAT-3 antibody (Cell Signaling) as per the manufacturer's instructions. Chain-like collections of positive cells are observed emanating radially from the ventricles in both poly(I:C)-treated and control embryos. It is unclear whether these cells are migrating neurons, or endothelial cells. Quantification of staining intensity tends to show more staining in the poly(I:C)-exposed embryos, but the difference is not significant. However, compared to the poly(I:C)-exposed embryos there is a high level of background STAT-3 staining in

the control embryos. Moreover, staining is inconsistent between embryos from the same treatment group. Therefore, we conclude that this method is not appropriate for measuring the small changes that we predict to occur in poly(I:C)-exposed embryos.

Next, we looked for signs of STAT-3 activation using Western blots of whole head homogenates at 0.5, 3 and 6 hours following maternal poly(I:C) administration. While still frozen, heads were dissociated in PBS/T containing protease and phosphatase inhibitors (Roche). Protein concentration was measured with a BSA assay and 50 μg protein was loaded onto an SDS-PAGE gel for Western blotting. Using similar protocols, we also analyzed lysates from placentas and from IL-6-treated BeWo placental cells. While we were able to detect pSTAT-3 in both BeWo cell lysates and in poly(I:C)-treated placentas (see Appendix B), we were not able to detect p-STAT-3 in fetal brains.

Finally, we reasoned that perhaps our inability to detect p-STAT-3 may be due to the short temporal window of activation. Tissue culture experiments demonstrate a rapid activation of STAT-3 following cytokine administration, followed by an equally rapid disappearance, often within 30 minutes. Alternatively, perhaps a small rise in p-STAT-3 is below the limit of detection of our Western blots, but is nevertheless biologically relevant. Therefore, we used RT-PCR to quantify the expression of a gene that is up-regulated by IL-6 activity, SOCS3. We extracted mRNA from fetal brains using the RNEasy kit (Qiagen), followed by DNAse treatment and reverse transcription using random decamer primers (Retroscript, Ambion) and PCR for SOCS3 (F: ACCAGCGCCACTTCTTCACG R: GTGGAGCATCATACTGATCC) and GAPDH (F: TTGCCATCAATGACCCCTTCA R: CGCCCCACTTGATTTTGGA). In a

preliminary experiment, we found an increase in SOCS3 mRNA expression after poly(I:C) injection (Fig. 2B).

Future Directions

Despite our inability to detect p-STAT-3 in the fetal brain, direct action of IL-6 on the fetal brain remains a viable hypothesis. We and others (Golan et al., 2005; Liverman et al., 2006; Meyer et al., 2006a; Meyer et al., 2006b; Meyer et al., 2007) have detected IL-6 in the fetal brain after MIA. Moreover, we have preliminary evidence that at least one IL-6 responsive gene, SOCS3, is upregulated in the fetal brain following MIA. Further work will focus on localizing the activation of several IL-6 responsive genes in the fetal brain. Identification of specific IL-6-responsive cell types will aid in delineating the molecular and anatomical pathways that underlie the behavioral abnormalities observed in MIA offspring. Both fetal brain and placenta continue to be potential targets of induced maternal IL-6.

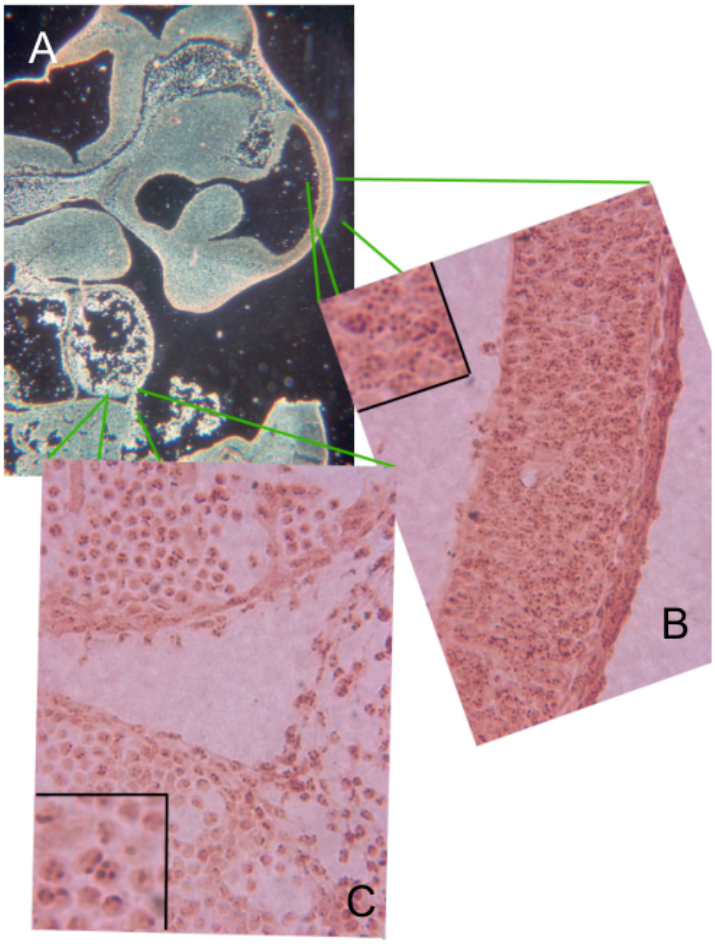
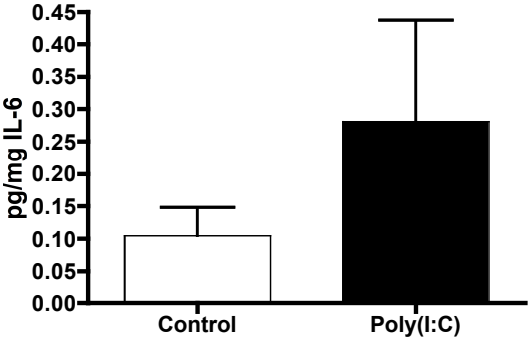


Figure 1. The IL-6 receptor is expressed throughout the E12.5 mouse embryo. (A) A sagital section of an E12.5 fetus is shown stained with anti-IL-6R. (B) In the developing cortex, punctate IL-6R staining is visible at a frequency of several puncta per cell. (C) Immuno-staining for IL-6R is also present in the majority of cells in the fetal heart, blood and liver.

A)



B)

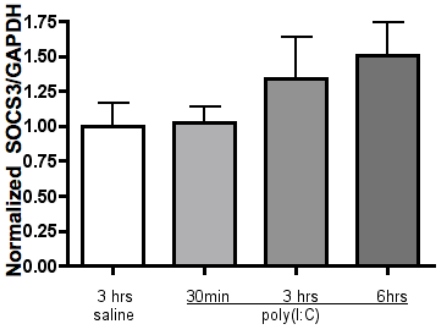


Figure 2. Detection of IL-6 and SOCS3 in the fetal brain following MIA. (A) IL-6 levels, measured by ELISA and expressed as pg of IL-6 per mg of fetal brain protein, are increased in the fetal brain three hours after MIA. (B) RT-PCR shows SOCS3 mRNA is elevated in the fetal brain beginning 30 minutes after MIA, and continuing for at least six hours. However, data from these pilot experiments are not significantly different.

Modified IL-6 ELISA protocol

For use with R&D IL-6 Duo-set ELISA kit, this protocol assumes that the reagents in the kit are reconstituted and as instructed by the manufacturer.

1) Add 55 µl capture antibody to 10 ml carbonate coating buffer (.025M NaCarbonate, .025M NaBiCarb, pH = 9.7) and coat wells overnight (ON) at 4C
2) Wash with PBS/0.05% Tween20 (PBS/T) 5 times
3) Block wells with 4% BSA/10% NGS in PBS 2hr (block solution)

--FOR ALL FURTHER DILUTIONS, USE 1:4 BLOCK SOLUTION DILUTED IN PBS/T (.25Blk)--

4) For standard curve, add 580 µl .25blk to 20 µl standard stock to yield 1000 pg/ml HI standard, 8 serial dilutions to 7.8 pg/ml LOW standard
5) Add 50 µl .25Blk to all but left 2 rows of plate
6) Add 100 µl standards in duplicate to left 2 rows; 50 µl of .25Blk to next 2 for blank
7) Add 50 µl sample to each well in duplicate; 2 hr room temp (RT) or ON 4^{o} C
8) Wash 5X PBS/T
9) Dilute 55 µl detection Ab in 10 ml .25Blk, 100 µl per well, RT for 2 hr
10) Wash 5X PBS/T

11) Strep-HRP 1:200 in .25Blk 45min RT

12) Wash 5X PBS/T

13) Develop R&D ELISA development reagents A+B

14) Wait 1/2 hr, stop with 2N (1M) H_2SO_4

15) Read using plate reader; software settings: lm1: 450 lm2:540; Reduction lm1-lm2; 4PL curve fit for standard curve.

References

Bauer S, Kerr BJ, Patterson PH (2007) The neuropoietic cytokine family in development, plasticity, disease and injury. Nat Rev Neurosci 8:221-232.

Dahlgren J, Samuelsson AM, Jansson T, Holmang A (2006) Interleukin-6 in the maternal circulation reaches the rat fetus in mid-gestation. Pediatr Res 60:147-151.

Dame JB, Juul SE (2000) The distribution of receptors for the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 in the developing human fetus. Early Hum Dev 58:25-39.

Gadient RA, Otten U (1994) Expression of interleukin-6 (IL-6) and interleukin-6 receptor (IL-6R) mRNAs in rat brain during postnatal development. Brain Res 637:10-14.

Gadient RA, Otten UH (1997) Interleukin-6 (IL-6)--a molecule with both beneficial and destructive potentials. Prog Neurobiol 52:379-390.

Golan HM, Lev V, Hallak M, Sorokin Y, Huleihel M (2005) Specific neurodevelopmental damage in mice offspring following maternal inflammation during pregnancy. Neuropharmacology 48:903-917.

Liverman CS, Kaftan HA, Cui L, Hersperger SG, Taboada E, Klein RM, Berman NE (2006) Altered expression of pro-inflammatory and developmental genes in the fetal brain in a mouse model of maternal infection. Neurosci Lett 399:220-225.

McLoughlin RM, Jenkins BJ, Grail D, Williams AS, Fielding CA, Parker CR, Ernst M, Topley N, Jones SA (2005) IL-6 trans-signaling via STAT3 directs T cell infiltration in acute inflammation. Proc Natl Acad Sci U S A 102:9589-9594.

Meyer U, Feldon J, Schedlowski M, Yee BK (2006a) Immunological stress at the maternal-foetal interface: a link between neurodevelopment and adult psychopathology. Brain Behav Immun 20:378-388.

Meyer U, Murray PJ, Urwyler A, Yee BK, Schedlowski M, Feldon J (2007) Adult behavioral and pharmacological dysfunctions following disruption of the fetal brain balance between pro-inflammatory and IL-10-mediated anti-inflammatory signaling. Mol Psychiatry.

Meyer U, Nyffeler M, Engler A, Urwyler A, Schedlowski M, Knuesel I, Yee BK, Feldon J (2006b) The time of prenatal immune challenge determines the specificity of inflammation-mediated brain and behavioral pathology. J Neurosci 26:4752-4762.

Ringheim GE, Szczepanik AM, Petko W, Burgher KL, Zhu SZ, Chao CC (1998) Enhancement of beta-amyloid precursor protein transcription and expression by the soluble interleukin-6 receptor/interleukin-6 complex. Brain Res Mol Brain Res 55:35-44.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Ulfig N, Friese K (1999) Interleukin-6 receptor is highly expressed in the ganglionic eminence of the human fetal brain. Biol Neonate 76:320-324.

Zaretsky MV, Alexander JM, Byrd W, Bawdon RE (2004) Transfer of inflammatory cytokines across the placenta. Obstet Gynecol 103:546-550.

Appendix F

Sustained, Biologically-Relevant Levels of Cytokine Expression in the Serum of Mice Using *In Vivo* Electroporation

Stephen Smith and Paul H. Patterson

Division of Biology, California Institute of Technology, Pasadena, CA

Written by Stephen Smith for inclusion in his thesis, Spring 2008

Rationale

During experimental influenza infection, cytokines are elevated in mouse serum for several days. Preliminary ELISAs for several cytokines showed elevated levels of 10-200 pg/ml in the serum persisting for several days. In order to determine which cytokine might be important for mediating the effects of maternal immune activation (MIA) on the embryo, we wanted to administer similar levels of several cytokines independently, in the absence of infection (see Chapter 3). However, these low, sustained levels of cytokines are difficult to mimic using traditional methods; injection of recombinant cytokines can produce any desired serum cytokine level, but the serum half-life of injected cytokines is on the order of several minutes (Castell et al., 1988). Repeated injection, or implantation of an osmotic minipump that continually releases small amounts of cytokines, are possible alternatives. However, these methods induce a stress response in the mouse and are therefore not suitable for use in the MIA model. Maternal stress is itself a risk factor for schizophrenia (Koenig et al., 2002) and the stress response can alter fetal development (Relier, 2001; Weinstock, 2001) as well as the maternal immune response (McEwen et al., 1997; Padgett et al., 2000; Moynihan, 2003). In fact, our early experiments demonstrated that the offspring of mice infected with influenza virus and subjected to daily saline injections did not develop the behavior deficits normally associated with maternal influenza infection (data not shown). Therefore, we needed to develop a method to produce a sustained serum cytokine elevation in a pregnant mouse, in the absence of infection and with minimal stress.

Nonviral gene transfer is an attractive technique for gene therapy, and several groups have reported high levels of protein expression from plasmids injected into

skeletal muscle followed by electroporation (Mir et al., 1999; Nakano et al., 2001; Adachi et al., 2002; Dona et al., 2003; Lu et al., 2003). Skeletal muscle has a number of unique characteristics that enable it to function as a "protein factory": an abundant supply of blood allows for easy export of product; the multi-nucleated fibers persist for much of the lifetime of the individual; the continuous nature of the fiber allows for dispersion of the transgene along the length of the muscle, far from the site of injection; if complications arise, a muscle can be removed with relatively minor complications, compared to other commonly targeted organs such as lungs, liver, etc. (Lu et al., 2003). Delivery of cDNA coding for interleukin-1 receptor antagonist (IL-1ra) or a viral interleukin-10 (vIL-10) construct using electroporation of muscle has been demonstrated to improve symptoms of myocarditis in mice (Nakano et al., 2001; Adachi et al., 2002), demonstrating the validity of this method of cytokine delivery. Finally, because the technique requires only a single brief injection and electroporation, during which the mouse is briefly anesthetized, the lack of stress makes this delivery method optimal for the MIA model. We planned to induce cytokine expression in pregnant rodents using electroporation, and screen for behavioral deficits in the offspring.

Methods and Results

We began by obtaining the plasmid vector pCAGGS (Niwa et al., 1991) that had previously been shown to promote expression cytokines in the mouse (Adachi et al., 2002; Yamakami et al., 2002; Zou et al., 2003) (CABRI, www.cabri.org), and pCAGGS with the gene for IL-6 cloned into the expression site (pCAmIL-6) (RIKEN Biorescource Center; Dr. Hirofumi Hamada). pCAmLIF, also from RIKEN, was used for some experiments to express leukemia inhibitory factor (LIF). pCALacZ was produced in-

house by inserting the LacZ gene from the plasmid shuttle-IRES-tau-lacZ(SITL) produced by Emma Dormand and Dr. David Anderson into the BglII site in pCAGGS. Plasmids were grown in *E. coli*, purified using the endotoxin-free Maxi kit (Qiagen), and concentrated to 1 μg/μl of plasmid DNA in PBS. Mice were mated overnight as previously described (Smith et al., 2007). On day 12.5 of pregnancy, mice were anesthetized with ketamine/xyzlazine, the right hind leg was shaved, and 50 μl of DNA solution was injected into the center of the tibialis anterior (TA) muscle. Visible swelling of the entire length of the muscle indicated the injection was successful. A pair of needle-like electrodes placed 5 mm apart (BTX 2-needle array Part#10-002603-01) was inserted into the TA muscle on either side of the injection site, lengthwise with the muscle. Inserting a new 25G needle at the site of electrode entry prior to insertion of the electrode facilitated electrode insertion. Six pulses of 40 msec duration at 100V (200 V/cm) were applied with an inter-pulse interval of 200 msec, using a BTX T820 square electroporated. Despite being under deep anesthesia, the mouse visibly twitched during electroporation.

Several methods were used to confirm transgene expression in non-pregnant mice. First, TA muscles were dissected three days after electroporation. Some muscles showed visible damage, notably reddening or swelling around the sites of electrode insertion. RNA was extracted using Tirol reagent and RT-PCR for IL-6 was preformed (F: GCAGCAGGTCCAACTGTGCTATCT R: TGGGTCTTCATCAGTTTCACAGCC). pCAmIL-6 electroporated animals showed marked increase in IL-6 mRNA expression, indicating strong expression of the transgene, while pCAGGS or pCAmLIF injected animals showed low levels of IL-6 expression, probably reflecting inflammation due to

tissue damage induced during the procedure (Fig. 1A). To visualize transgene expression, pCALacZ was electroporated into TA muscle, and three days later mice were sacrificed and whole-mount leg preparations were prepared. Skin was removed and legs were fixed in 4% PFA for 30 min, followed by an overnight rinse in PBS. Legs were incubated in a solution containing the following at 37^{o} C for 3 hrs: 25 mm potassium ferrocyanide, 25 mm potassium ferricyanide, 2 mm $MgCl_2$, 0.02% NP50, 0.1% sodium deoxycholate in PBS. Pictures were taken on a dissection microscope (Fig. 1 B,C). At higher magnification, individual $LacZ^{+}$ fibers can be seen (Fig. 1D).

Further experiments confirmed the secretion of biologically active IL-6 into the serum of electroporated animals. Serum was collected at various time points after electroporation, and an ELISA for IL-6 was preformed according to the manufacture's instructions (see Appendix F for detailed protocol). In untreated animals, IL-6 was undetectable. Three days after electroporation, IL-6 levels increased, and a maximum level was seen at 7 days. By 10 days, IL-6 levels had begun to decrease; however, even 31 days after electroporation (a time point which corresponds to the day of weaning the pups of a mouse electroporated on E12.5 of pregnancy), the IL-6 level in the serum was significantly elevated (Fig. 2A). Upon dissection, the spleens of IL-6 treated animals were noticeably larger than those of control animals, averaging 0.23g and 0.10g respectively. Moreover, the weight of the spleens correlated with the level of IL-6 found in the serum of the same animal (Fig. 2B, $r^2 = 0.86$, $p < 0.05$). Splenomegaly has previously been associated with hyperactive IL-6 signaling (Jenkins et al., 2007).

We then allowed mice electroporated on E12.5 of pregnancy to give birth and raise their litters as normal. When the offspring were weaned at three weeks, we noted

that the offspring of pCAmIL-6-treated mothers had hair loss covering large portions of their body, which was markedly different from pCAGGS-treated mice (Fig. 3). The pattern of hair loss, large, poorly-defined bald areas with sparse, patchy hairs remaining, does not match the pattern observed as a result of grooming, namely completely bald areas with no fur remaining and with well-defined borders. A few, seemingly contradictory reports exist in the literature concerning IL-6 and hair growth. IL-6 has been reported to induce hair follicle growth (Tanabe et al., 2006), yet IL-6 and the IL-6 family member Oncostatin M have been reported to inhibit hair growth (Yu et al., 2008). This observation of hair loss in the pups, coupled with our unpublished observation of hair loss in IL-6 knockout mice, suggests that IL-6 plays a role in hair growth or loss. Barbering is an unlikely explanation for the hair loss because the pattern of hair loss did not resemble that seen from barbering. Moreover, the hair re-grew in the pups after weaning, suggesting that the maternal milk carried some factor that reversibly caused hair loss. Published studies in humans (Canard et al., 2007) and pigs (Nguyen et al., 2007) indicated that cytokines can transfer from mother to offspring in milk. This suggests the possibility that postnatal, rather than prenatal cytokine exposure could cause behavioral deficits in these animals. Experiments injecting cytokines into young, postnatal animals demonstrated that postnatal cytokine exposure can cause behavior deficits in the adult animals (Tome et al., 2004; Watanabe et al., 2004).

Therefore, we used a cross-fostering paradigm in which offspring from pCAGGS-electroporated mothers were switched with either offspring from another pCAGGS mother, or with offspring from pCAmIL-6 electroporated (and vice-versa) mother soon after birth. Thus, we had four groups that were behaviorally tested, defined by the born

to/raised by treatment: control/control, control/IL-6, IL-6/control and IL-6/IL-6 (N = 14, 29, 23, 15). As shown in Fig. 4, IL-6/IL-6 animals showed significant reductions in PPI compared to control/control animals, indicating that maternal electroporation with pCAmIL-6 had a significant effect on the behavior of the offspring. However, the cross-fostering experiment revealed that prenatal IL-6 had a non-significant effect if the pup was raised by a control mother. Conversely, control pups raised by pCAmIL-6 mothers showed significant PPI deficits compared to controls, and compared to mice born to pCAmIL-6 mothers and raised by control mothers. Two-way ANOVA with prenatal and postnatal treatment as the variables revealed a significant effect of postnatal treatment with no effect of prenatal treatment for PPI(15) ($p < 0.001$), a similar trend towards significance for PPI(5) ($p < 0.06$). Similar results were obtained using BALB/c mice in a separate experiment. Control/IL6 (N = 40) had significantly lower PPI (15) than control/control animals (N = 17) ($p > 0.05$) and a trend towards significance at PPI (5) ($p = 0.2$). IL-6/control animals (N = 45) were not significantly different than either control/control ($p = 0.79$) or control/IL6 animals ($p = 0.17$).

Conclusions

The observation that postnatal exposure to IL-6 causes PPI deficits is interesting, and is consistent with other cross-fostering experiments by our group using maternal influenza infection (L. Shi, unpublished) and by others using maternal poly(I:C) (Meyer et al.) However, those studies also found an effect of MIA (prenatal) on behavior of the offspring. That is, they concluded that both pre- and postnatal experiences are relevant for offspring behavior. There is no published data suggesting that maternal sickness while breastfeeding predisposes the infant to mental disease. NIH guidelines suggest

continuing breastfeeding during an illness, unless the mother is taking medication that may be harmful to the infant (Oreille, 2007). However, several studies involving cytokine injection into neonatal mice have found behavioral changes in the offspring that are similar to those seen in the MIA model (Tohmi et al., 2004; Watanabe et al., 2004), and cytokines can be transferred via breast milk (Nguyen et al., 2007; Canard et al., 2007). Thus, the transfer of cytokines via the breast milk warrants further investigation.

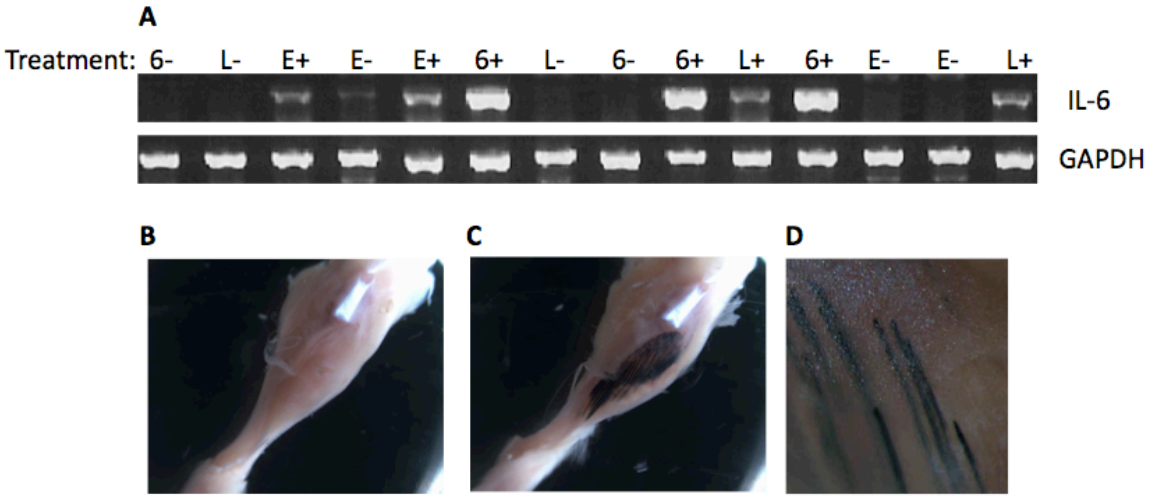


Figure 1: IL-6 is expressed in the tibialis anterior muscle following electroporation with pCAmIL-6. (A) RT-PCR assay for IL-6 and GAPDH on RNA isolated from the TA muscle of mice electroporated with pCAmIL-6 (lanes labeled "6"), pCAmLIF ("L") and empty vector pCAGGS ("E"). A + indicates RNA from the electroporated leg, while a - indicates the contralateral, untreated leg. Whole-mounts of legs treated with pCAGGS (B) or pCALacZ (C,D) show that individual electroporated muscle fibers express LacZ.

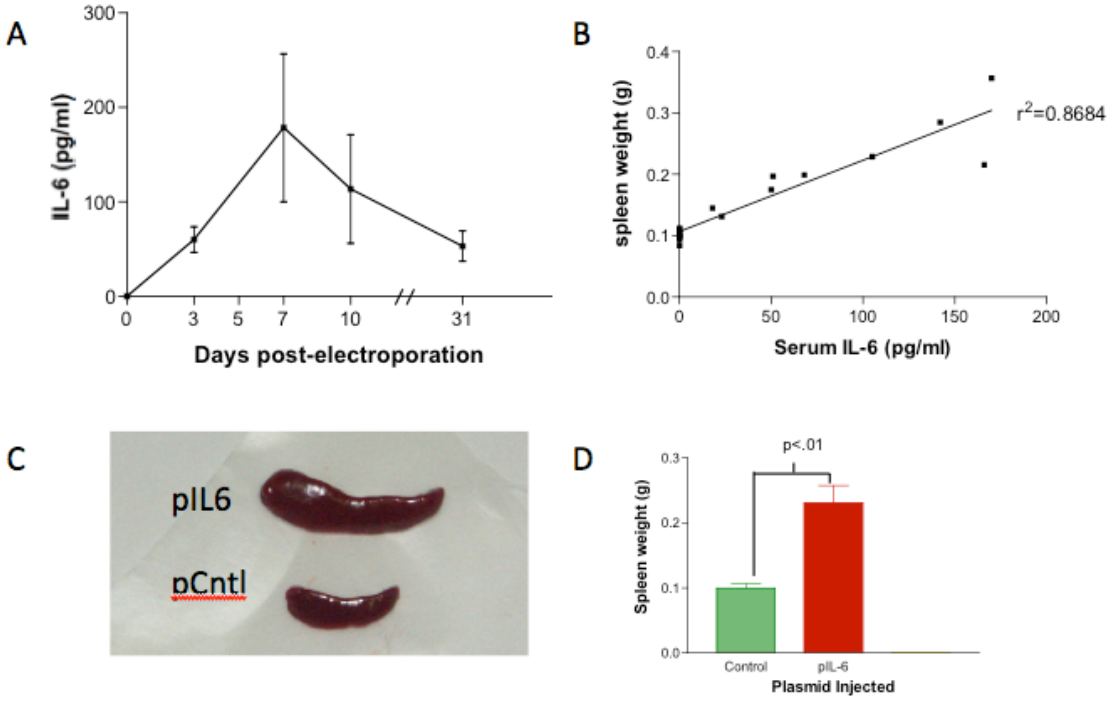


Figure 2. Biologically active IL-6 is found in the blood of pCAmIL-6 electroporated mice. (A) IL-6 is found in the serum by ELISA. Expression peaks at about 7 days post-electroporation, and persists until at least 31 days. (B) Spleen weight is correlated with serum IL-6 levels of electroporated mice. $P < 0.05$. (C) A representative picture is shown of spleens from pCAmIL-6 electroporated and control (pCntl) mice. (D) Spleens of IL-6 electroporated mice are significantly heavier than controls.

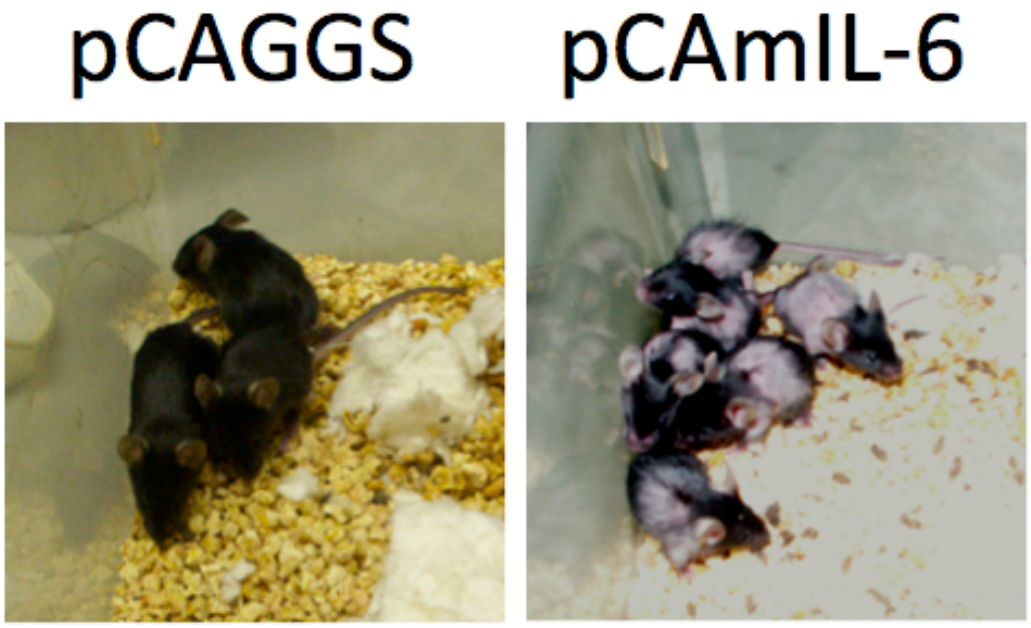


Figure 3. Offspring of pCAmIL-6 mice show significant hair loss at weaning. In the offspring of pCAmIL-6 mothers, hair loss is patchy, with long, wispy hairs visible in the bald areas. This is not the pattern of hair loss usually associated with barbering, where all of the hair in a well-defined area is missing.

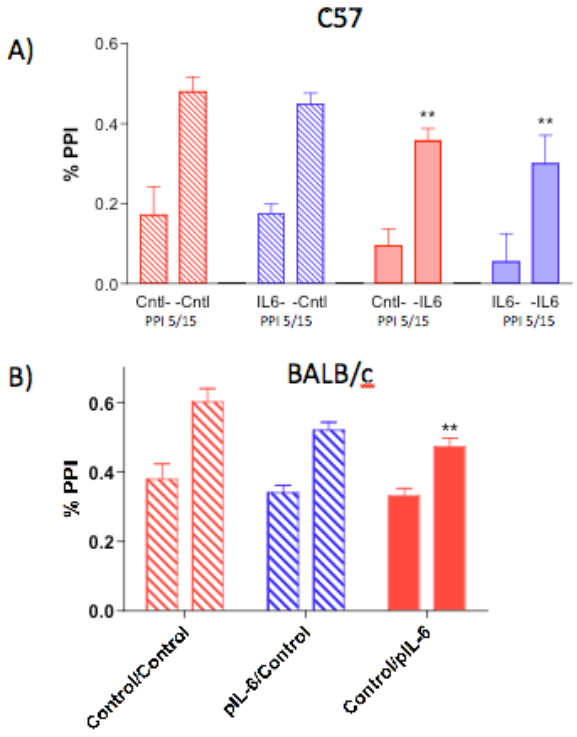


Figure 4. The offspring of electroporated mice display PPI deficits. The first of the two bars in each pair represents PPI in response to a 5 db stimulus and the second bar represents PPI in resonse to 15 db. (A) Compared to the offspring of control mice raised by control mothers, the offspring of pCAmIL-6 mice raised by pCAmIL-6 mothers show a significant PPI deficit, as do control offspring raised by pCAmIL-6 mothers. However, mice born to pCAmIL-6 mother but raised by control mothers do not show a significant PPI deficit. Two-way ANOVA with prenatal treatment and postnatal treatment as variables reveled a significant effect of postnatal environment, with no effect of prenatal treatment and no interaction. (B) Similar results were obtained in BALB/c mice. ** $p < 0.05$ compared to control/control.

References

Adachi O, Nakano A, Sato O, Kawamoto S, Tamara H, Toyoda N, Yamato E, Matsumori A, Tabayashi K, Miyazaki J (2002) Gene transfer of Fc-fusion cytokine by in vivo electroporation: application to gene therapy for viral myocarditis. Gene Ther 9:577-583.

Castell JV, Geiger T, Gross V, Andus T, Walter E, Hirano T, Kishimoto T, Heinrich PC (1988) Plasma clearance, organ distribution and target cells of interleukin-6/hepatocyte-stimulating factor in the rat. Eur J Biochem 177:357-361.

Dona M, Sandri M, Rossini K, Dell'Aica I, Podhorska-Okolow M, Carraro U (2003) Functional in vivo gene transfer into the myofibers of adult skeletal muscle. Biochem Biophys Res Commun 312:1132-1138.

Jenkins BJ, Roberts AW, Greenhill CJ, Najdovska M, Lundgren-May T, Robb L, Grail D, Ernst M (2007) Pathologic consequences of STAT3 hyperactivation by IL-6 and IL-11 during hematopoiesis and lymphopoiesis. Blood 109:2380-2388.

Koenig JI, Kirkpatrick B, Lee P (2002) Glucocorticoid hormones and early brain development in schizophrenia. Neuropsychopharmacology 27:309-318.

Lu QL, Bou-Gharios G, Partridge TA (2003) Non-viral gene delivery in skeletal muscle: a protein factory. Gene Ther 10:131-142.

McEwen BS, Biron CA, Brunson KW, Bulloch K, Chambers WH, Dhabhar FS, Goldfarb RH, Kitson RP, Miller AH, Spencer RL, Weiss JM (1997) The role of

adrenocorticoids as modulators of immune function in health and disease: neural, endocrine and immune interactions. Brain Res Brain Res Rev 23:79-133.

Mir LM, Bureau MF, Gehl J, Rangara R, Rouy D, Caillaud JM, Delaere P, Branellec D, Schwartz B, Scherman D (1999) High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci U S A 96:4262-4267.

Moynihan JA (2003) Mechanisms of stress-induced modulation of immunity. Brain Behav Immun 17 Suppl 1:S11-16.

Nakano A, Matsumori A, Kawamoto S, Tahara H, Yamato E, Sasayama S, Miyazaki JI (2001) Cytokine gene therapy for myocarditis by in vivo electroporation. Hum Gene Ther 12:1289-1297.

Nguyen TV, Yuan L, Azevedo MS, Jeong KI, Gonzalez AM, Saif LJ (2007) Transfer of maternal cytokines to suckling piglets: in vivo and in vitro models with implications for immunomodulation of neonatal immunity. Vet Immunol Immunopathol 117:236-248.

Niwa H, Yamamura K, Miyazaki J (1991) Efficient selection for high-expression transfectants with a novel eukaryotic vector. Gene 108:193-199.

O'Reiley D (2007) Overcoming breastfeeding problems. http://wwwnlmnihgov/medlineplus/ency/article/002452htm.

Padgett DA, Loria RM, Sheridan JF (2000) Steroid hormone regulation of antiviral immunity. Ann N Y Acad Sci 917:935-943.

Relier JP (2001) Influence of maternal stress on fetal behavior and brain development. Biol Neonate 79:168-171.

Smith SE, Li J, Garbett K, Mirnics K, Patterson PH (2007) Maternal immune activation alters fetal brain development through interleukin-6. J Neurosci 27:10695-10702.

Tanabe A, Ogawa Y, Takemoto T, Wang Y, Furukawa T, Kono H, Adachi Y, Kusumoto K (2006) Interleukin 6 induces the hair follicle growth phase (anagen). J Dermatol Sci 43:210-213.

Tohmi M, Tsuda N, Watanabe Y, Kakita A, Nawa H (2004) Perinatal inflammatory cytokine challenge results in distinct neurobehavioral alterations in rats: implication in psychiatric disorders of developmental origin. Neurosci Res 50:67-75.

Watanabe Y, Hashimoto S, Kakita A, Takahashi H, Ko J, Mizuno M, Someya T, Patterson PH, Nawa H (2004) Neonatal impact of leukemia inhibitory factor on neurobehavioral development in rats. Neurosci Res 48:345-353.

Weinstock M (2001) Alterations induced by gestational stress in brain morphology and behaviour of the offspring. Prog Neurobiol 65:427-451.

Yamakami K, Akao S, Tadakuma T, Nitta Y, Miyazaki J, Yoshizawa N (2002) Administration of plasmids expressing interleukin-4 and interleukin-10 causes BALB/c mice to induce a T helper 2-type response despite the expected T helper 1-type response with a low-dose infection of Leishmania major. Immunology 105:515-523.

Yu M, Kissling S, Freyschmidt-Paul P, Hoffmann R, Shapiro J, McElwee KJ (2008) Interleukin-6 cytokine family member oncostatin M is a hair-follicle-expressed factor with hair growth inhibitory properties. Exp Dermatol 17:12-19.

Zanardo V, Golin R, Amato M, Trevisanuto D, Favaro F, Faggian D, Plebani M (2007) Cytokines in human colostrum and neonatal jaundice. Pediatr Res 62:191-194.

Zou Y, Takano H, Mizukami M, Akazawa H, Qin Y, Toko H, Sakamoto M, Minamino T, Nagai T, Komuro I (2003) Leukemia inhibitory factor enhances survival of cardiomyocytes and induces regeneration of myocardium after myocardial infarction. Circulation 108:748-753.

Appendix G

Retrotransposon Activation in Embryonic Brain Following Maternal Immune Activation

Alysson Muotri[1], Stephen Smith[2], Paul H. Patterson[2] and Fred Gage[1]

[1] Laboratory of Genetics, The Salk Institute for Biological Studies, La Jolla, CA

[2] Division of Biology, California Institute of Technology, Pasadena, CA

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Rationale

Approximately 44% of the human genome is comprised of transposons and retrotransposons. Once considered "junk DNA" or selfish genes, these elements are now recognized as having important regulatory functions (Muotri and Gage, 2006; Muotri et al., 2008). Long interspersed nucleotide elements-1 (LINE-1, or L1) alone comprise about 20% of the mammalian genome, although only a small portion of these retroelements are able to transpose (Muotri and Gage, 2006; Muotri et al., 2008). When they do activate, however, these elements are able to promote not only L1 transposition, but also the transposition of other types of elements in *trans* (Muotri and Gage, 2006; Muotri et al., 2008). Thus, L1 elements have the potential to mobilize large stretches of the genome and create insertions of various elements that have the potential to affect gene expression.

The mobilization of L1 retrotransposons could play an important role in generating the immense diversity observed in the central nervous system (CNS) (Muotri and Gage, 2006). L1 elements mobilize in both human and mouse neuronal stem cells, but not in many other types of somatic stem cells (Muotri et al., 2005; Garcia-Perez et al., 2007). Several lines of transgenic mice in which L1 retrotransposition events activate a GFP transgene indicate that L1 retrotransposition is highly active during brain development, but not during the development of other tissue types (Muotri et al., 2005). Further, the insertion of retrotransposons into the genome is not random. In mice, L1 retrotransposition events seem to occur at "hot-spots" near neuronal genes, and can alter expression of those genes (Muotri et al., 2005; Chen et al., 2006; Muotri et al., 2008). In yeast, the Ty1 transposon is inserted upstream of tRNA genes in a highly nonrandom

fashion (Bachman et al., 2004). Thus, retrotransposition may generate a genetic mosaic in the brain by activating L1 elements specifically in neuronal precursors, and these elements then insert themselves specifically into neuronal genes. This would enable neighboring neurons to be genetically and functionally different.

Retrotransposition may also be altered in mental disease. In Mecp2 mutant mice and in humans with Rett syndrome, there is a higher level of retrotransposition compared to normal controls. While this may be a simple byproduct of the MecP2 mutation causing a non-specific lack of transcriptional repression, it could also reflect a fundamental deficit of the disease (Muotri, personal communication). In addition, high levels of endogenous retroviruses are associated with schizophrenia (Karlsson et al., 2001; Karlsson et al., 2004; Yolken et al., 2008). Thus, it is possible that, while normal levels of retrotransposition generate diversity, too many retrotransposition events are maladaptive. Although there is correlative evidence suggesting that retrotransposition may change behavior (Muotri et al., 2005; Yolken et al., 2008), a direct link between retrotransposition and behavior has yet to be experimentally demonstrated.

Preliminary results

We hypothesized that maternal immune activation (MIA) might activate L1 retrotransposition in mice for two reasons. First, stress signals such as those generated during immune challenge activate endogenous retroviral elements (Cho et al., 2008). Further, some of the prenatal infections that are associated with schizophrenia have also been associated with endogenous retroviral activation (for example, *Toxoplasma gondii* (Brown et al., 2005; Frank et al., 2006)). Second, the MIA model of schizophrenia has

high construct and face validity (Patterson, 2002; Fatemi, 2005), and endogenous retroviruses are activated in schizophrenia (Yolken et al., 2008). Therefore, we established a collaboration with Fred Gage and Alysson Muotri who are conducting experiments on L1 retrotransposition in the mouse brain. We injected pregnant transgenic mice in which L1 retrotransposition events activate a GFP transgene with poly(I:C) on E12.5 of pregnancy. We then sacrificed several adult offspring, each from different litters, for analysis (N = 5 for control and 7 for poly(I:C)). We sectioned the entire brain of the adult offspring, collected every 6^{th} section, and counted the number of GFP^+ cells in each section. Data are expressed as number of GFP^+ cells per section in each animal, calculated as the average of 15 sections per animal. Differences between groups are compared with T-tests, where N is the number of animals, not the total number of sections. Animals treated with poly(I:C) display a highly significant, three-fold increase of $EGFP^+$ cells in the brain (Fig. 1, $p < 0.001$).

Future Directions

These pilot results indicate that MIA is able to induce increased L1 retrotransposition in the brains of the offspring. We now hope to relate the behavior of the offspring (see Chapters 2 and 3) with retrotransposition, to determine if retrotransposon activation is simply a by-product of MIA, or contributes to the abnormal behavior seen in MIA offspring. We plan to use L1 mice in four experimental groups: (1) control, saline-injected, (2) MIA induced by poly(I:C), (3) MIA induced by poly(I:C) and co-injected with an anti-IL-6 antibody, and (4) MIA induced by poly(I:C) followed by treatment with an inhibitor of retrotransposition. We will then analyze both the behavior of the offspring and the number of $EGFP^+$ cells in the brain. Will a treatment that we know prevents

behavioral deficits (Group 3), also inhibit L1 activation? Conversely, will a drug that prevents L1 activation prevent behavioral deficits in the offspring? This experiment could address the question of whether retrotransposition is able to alter the behavior of the organism.

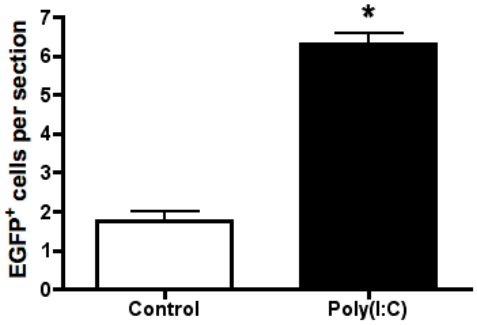

Figure 1. L1 activation is increased in the brains on MIA adult offspring.

Every 6th section was collected across the entire brain, and $EGFP^+$ cells were counted. A three-fold increase in $EGFP^+$ cells was observed in the offspring of poly(I:C) treated mice. N = 7 control, 5 poly(I:C) animals, * $p < 0.001$.

References

Bachman N, Eby Y, Boeke JD (2004) Local definition of Ty1 target preference by long terminal repeats and clustered tRNA genes. Genome Res 14:1232-1247.

Brown AS, Schaefer CA, Quesenberry CP, Jr., Liu L, Babulas VP, Susser ES (2005) Maternal exposure to toxoplasmosis and risk of schizophrenia in adult offspring. Am J Psychiatry 162:767-773.

Chen J, Rattner A, Nathans J (2006) Effects of L1 retrotransposon insertion on transcript processing, localization and accumulation: lessons from the retinal degeneration 7 mouse and implications for the genomic ecology of L1 elements. Hum Mol Genet 15:2146-2156.

Cho K, Lee YK, Greenhalgh DG (2008) Endogenous Retroviruses in Systemic Response to Stress Signals. Shock.

Fatemi SH (2005) Prenatal human influenza viral infection, brain development and schizophrenia. in Neuropsychiatric disorders and infeciton (ed S H Fatemi) pp 66-83 Taylor and Francis, UK.

Frank O, Jones-Brando L, Leib-Mosch C, Yolken R, Seifarth W (2006) Altered transcriptional activity of human endogenous retroviruses in neuroepithelial cells after infection with Toxoplasma gondii. J Infect Dis 194:1447-1449.

Garcia-Perez JL, Marchetto MC, Muotri AR, Coufal NG, Gage FH, O'Shea KS, Moran JV (2007) LINE-1 retrotransposition in human embryonic stem cells. Hum Mol Genet 16:1569-1577.

Karlsson H, Schroder J, Bachmann S, Bottmer C, Yolken RH (2004) HERV-W-related RNA detected in plasma from individuals with recent-onset schizophrenia or schizoaffective disorder. Mol Psychiatry 9:12-13.

Karlsson H, Bachmann S, Schroder J, McArthur J, Torrey EF, Yolken RH (2001) Retroviral RNA identified in the cerebrospinal fluids and brains of individuals with schizophrenia. Proc Natl Acad Sci U S A 98:4634-4639.

Muotri AR, Gage FH (2006) Generation of neuronal variability and complexity. Nature 441:1087-1093.

Muotri AR, Marchetto MC, Gage FH (2008) From the "RNA world" to brain complexity: generation of diversity. in Retrotransposition, diversity and the brain (Eds Gage, F H and Christen, Y) pp 53-64 Springer, NY.

Muotri AR, Chu VT, Marchetto MC, Deng W, Moran JV, Gage FH (2005) Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition. Nature 435:903-910.

Patterson PH (2002) Maternal infection: window on neuroimmune interactions in fetal brain development and mental illness. Curr Opin Neurobiol 12:115-118.

Yolken RH, Karlsson H, Bossis I, L. A, Dickerson F, Nellaker C, Elashoff M, Rubalcaba E, Viscidi RP (2008) Endogenous retroviruses and human neuropsychiatric disorders. in Retrotransposition, diversity and the brain (Eds Gage, F H and Christen, Y) pp 53-64 Springer, NY.