

**BEHAVIORAL ALTERATIONS IN RAT OFFSPRING  
FOLLOWING MATERNAL IMMUNE ACTIVATION AND  
CXC CHEMOKINE RECEPTOR ANTAGONISM**

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By

Stephanie A. Ballendine

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## ABSTRACT

Schizophrenia patients typically exhibit cognitive impairments that directly affect their daily functioning, but are not effectively treated by current antipsychotics. Maternal immune activation (MIA) during pregnancy, which can be triggered by a variety of infectious agents, has been associated with the development of schizophrenia in adult offspring. Epidemiological evidence indicates that elevated maternal levels of the chemokine interleukin- 8 (IL-8) during MIA contribute to the neurodevelopmental alterations underlying the disorder. The present experiments used an animal model of neurodevelopmental disorders to study the effects of MIA and chemokine receptor antagonism on the behavior of rat offspring, with behavioral tests chosen to examine cognitive functions that are typically impaired in human schizophrenia patients. The viral mimetic polyinosinic-polycytidylic acid (polyI:C) (4.0 mg/kg, i.v.) was injected into pregnant Long-Evans (LE) dams on gestational day (GD) 15. Dams were also treated with the three injections of CXCL8(3–72)K11R/G31P (G31P) (500 µg/kg, i.p.), a chemokine receptor antagonist that binds CXCR1 and CXCR2 with high affinity. PolyI:C treatment significantly increased maternal levels of the chemokine CXCL1, the rodent analogue of IL-8 that binds CXCR1 and CXCR2. The offspring of polyI:C-treated dams showed impaired associative recognition memory and multisensory integration, as well as subtle impairments in prepulse inhibition (PPI). G31P administration did not reverse any of the behavioral deficits caused by polyI:C, although G31P did alter PPI during adolescence. Although the present experiments included replications and novel findings for the polyI:C model, the effects of polyI:C were smaller than in other published research. Utilizing animal models that include both genetic and environmental

components, as well as more widely targeted anti-inflammatory therapies will likely result in more promising findings in future research.

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## LIST OF ABBREVIATIONS

DN-DISC1	dominant negative- disrupted in schizophrenia 1
DR	discrimination ratio
ED	embryonic day
fMRI	functional magnetic resonance imaging
G31P	CXCL8(3–72)K11R/G31P
GD	gestational day
IL-8	interleukin- 8
i.p.	intraperitoneal
i.v.	intravenous
LE	Long-Evans
lipopolysaccharide	LPS
MAM	methylazoxymethanol
MATRICES	Measurement and Treatment Research to Improve Cognition in Schizophrenia
MIA	maternal immune activation
mPFC	medial prefrontal cortex
OIP	object-in-place
PCP	phencyclidine
PFC	prefrontal cortex
PND	postnatal day
PolyI:C	polyinosinic-polycytidylic acid
PPC	posterior parietal cortex

PPI	prepulse inhibition
PRh	perirhinal cortex
sal	saline
SD	Sprague-Dawley
SEM	standard error of the mean
TNF- $\alpha$	tumor necrosis factor alpha

## INTRODUCTION

Schizophrenia is an extremely debilitating psychiatric disorder that causes significant health, social and financial burden for patients, their families and society as a whole (Knapp et al., 2004; Montgomery et al., 2013). Much of the debilitation experienced by individuals with schizophrenia is caused by the cognitive symptoms of the illness, which directly affect patients' daily functioning, ability to live independently and ability to work (Kitchen et al., 2012). These cognitive impairments are especially problematic, as current antipsychotics cannot treat them effectively (Wallace et al., 2012). A preventative treatment strategy that stops the neurodevelopmental alterations underlying schizophrenia from occurring will likely be more effective than any symptom-driven medication. Research investigating the mechanisms underlying schizophrenia led to the formation of the cytokine hypothesis, which postulates that abnormal cytokine elevations during pregnancy transmit inflammatory signals through the immature blood-brain barrier to the fetal brain, perturbing its development (Watanabe et al., 2010). Chemokines, a sub-class of cytokines with chemotactic properties that are primarily recognized for their role in leukocyte trafficking (Tran and Miller, 2003), are also being investigated for their role in these processes (Brown et al., 2004b; Arrode-Brusés and Brusés, 2012). Gaining a better understanding of how cytokines contribute to the neurodevelopmental alterations underlying schizophrenia will, hopefully, aid the development of more effective therapeutics in the future. Therefore, the aim of the present experiments was to use an animal model to examine the effects of maternal immune activation (MIA) and chemokine receptor antagonism during pregnancy on the cognitive functioning of rat offspring.

## **Inflammation and Schizophrenia**

Numerous studies have found evidence that maternal infection during pregnancy increases the offspring's risk of developing neurodevelopmental disorders, such as schizophrenia and autism (Brown et al., 2004a; Arias et al., 2012; Atladottir et al., 2012). The release of maternal and fetal cytokines during infection is thought to mediate the neurodevelopmental alterations underlying these disorders, as their occurrence is increased by a variety of infectious agents (Fineberg and Ellman, 2013). Significantly elevated plasma cytokine levels are also found in adult schizophrenia patients (Miller et al., 2011), suggesting that the inflammatory systems of these individuals have been dramatically altered. Studies examining anti-inflammatory drugs as adjuvant therapies for schizophrenia found modest symptom improvements following treatment with non-steroidal anti-inflammatory drugs (Nitta et al., 2013), minocycline (Levkovitz et al., 2010) and the cyclooxygenase-2 inhibitor celecoxib (Akhondzadeh et al., 2007; Müller et al., 2010), with one celecoxib trial finding no treatment effect (Rapaport et al., 2005). Girgis et al. (2014) postulated that these treatment effects could have been larger if the anti-inflammatory drugs were specifically targeted to cytokines. Administering drugs earlier in development, prior to the onset of symptoms, would also likely increase treatment effects.

Although numerous cytokines could be chosen for the design of a targeted anti-inflammatory therapy, several studies indicate that the chemokine interleukin-8 (IL-8) should be investigated. An epidemiological study conducted by Brown et al. (2004b) using maternal serum samples from a large birth cohort found a correlation between elevated serum levels of IL-8 during pregnancy and the development of schizophrenia

spectrum disorders in adult offspring. Further study of schizophrenia patients from the same birth cohort revealed correlations between prenatal exposure to elevated IL-8 and structural brain alterations that are typically seen in the disorder, including increased ventricular and decreased cortical volumes (Ellman et al., 2010). Coinciding with the finding of abnormal plasma cytokine levels in schizophrenia (Miller et al., 2011), the production of numerous chemokines by mononuclear cells from peripheral blood samples of human patients was also altered (Reale et al., 2011). Despite these intriguing findings, the range of effects of IL-8 and their underlying mechanisms are not entirely understood.

### **Animal Models of Schizophrenia**

The most practical method for investigating the developmental effects of cytokine elevations is rodent research. Modeling the entire spectrum of a complex human disorder such as schizophrenia in rodents is likely impossible, but different models can be used to examine various behavioral, physiological and neuroanatomical phenotypes of the disorder (Meyer and Feldon, 2012). An ideal model would exhibit high face, construct and predictive validity for the disorder it is designed to study. The earliest models of schizophrenia involved pharmacological manipulations, such as administration of amphetamine or phencyclidine (PCP). These drugs have been shown to increase locomotor activity and impair prepulse inhibition (PPI) in rodents, and exacerbate symptoms in human schizophrenia patients (McGonigle, 2014). The advantage of these models is their high predictive validity for treatment of the positive symptoms of schizophrenia, which include hallucinations and delusions. However, these models show low construct validity and low predictive validity for the other symptom domains (McGonigle, 2014).

Models with increased construct validity have focused on the developmental aspects of schizophrenia. Treating pregnant rats with methylazoxymethanol (MAM), a compound that inhibits neuroblast proliferation, induces anatomical and behavioral changes in the offspring that resemble schizophrenia (Moore et al., 2006). Another developmental model that has been used in research is the neonatal ventral hippocampal lesion model, in which ibotenic acid is injected into the ventral hippocampus of rodents on postnatal day (PND) 7. Ventral hippocampal lesions have also been shown to cause structural brain changes and behavioral abnormalities in the offspring that resemble schizophrenia (Jones et al., 2011). While the face validity of these models is relatively high, the construct validity is still not as high as desired, as MAM treatment and hippocampal lesions are not naturally occurring causes of schizophrenia.

The animal model chosen for the present experiments also focuses on the developmental aspects of schizophrenia. In this model, pregnant rodents are injected with the viral mimetic polyinosinic-polycytidylic acid (polyI:C). PolyI:C is synthetically manufactured double-stranded RNA that binds toll-like receptor 3 and elicits an inflammatory response (Doukas et al., 1994; Patterson, 2009). In mice, polyI:C treatment increases maternal serum levels of the cytokines IL-1 $\beta$ , IL-6, IL-10 and tumor necrosis factor alpha (TNF- $\alpha$ ) 3 h following treatment (Meyer et al., 2006), as well as serum levels of the chemokines Eotaxin, RANTES and MCP-1 on PND 7 in the offspring (Garay et al., 2013). One study in Sprague-Dawley (SD) rats used ELISA assays to show that IL-10 and TNF- $\alpha$  were significantly increased following polyI:C treatment (Song et al., 2011). However, ELISAs for other cytokines were not performed by Song et al. (2011), leaving the cytokine response of rats to polyI:C incompletely analyzed.

The offspring of polyI:C-treated rodents have been shown to exhibit behavioral impairments consistent with those seen in human schizophrenia, including impaired PPI, memory, set shifting and reversal learning (Zuckerman and Weiner, 2005; Wolff and Bilkey, 2008, 2010; Dickerson et al., 2010; Han et al., 2011; Howland et al., 2012; Zhang et al., 2012). These behavioral impairments likely arise from exposure to elevated cytokines, as cytokines are critical components of neurodevelopmental cell signaling pathways (Deverman and Patterson, 2009). Elevated levels of the cytokines IL-1 $\beta$  and TNF- $\alpha$  were also shown to reduce the number of dendritic nodes and total dendritic length in an *in vitro* study using cortical neurons from rats (Gilmore et al., 2004). Therefore, elevated cytokines likely affect behavior through their actions in multiple neurodevelopmental processes. The research outlined above highlighting the associations between inflammation and schizophrenia, and the behavioral changes exhibited by animals in the polyI:C model indicate that this model possesses high construct and face validity.

### **Chemokine Receptor Antagonism**

One aim of the present experiments was to examine the effects of IL-8 receptor antagonism during the acute inflammatory event triggered by polyI:C. Human IL-8 binds to CXCR1 and CXCR2, G-protein-coupled CXC chemokine receptors that are present in similar amounts on neutrophils (Baggiolini et al., 1997). When IL-8 binds CXCR1/CXCR2 it facilitates the migration of neutrophils from the bloodstream into inflamed tissues (Huber et al., 1991) and neutrophil activation (Zeilhofer and Schorr, 2000). While CXCR1 and CXCR2 are present in rodents, IL-8 is not produced. Rodent CXCL1 (also known as Gro/KC) is considered to be the murine ortholog of IL-8 as both



chemokines bind CXCR1 and CXCR2 and have inflammatory functions (Baggiolini et al., 1995; Zlotnik et al., 2006).

Research has shown that IL-8 and CXCL1 are also involved in neurodevelopmental processes. As mentioned above, prenatal exposure to elevated maternal serum levels of IL-8 was positively correlated with structural brain alterations that are typically seen in schizophrenia (Ellman et al., 2010). CXCL1 is critically involved in the development of central nervous system myelination, as it signals oligodendrocyte progenitors to stop migrating and proliferate once they have reached their destination (Robinson et al., 1998; Tsai et al., 2002). CXCL1 also affects neuronal development, as a study using cells obtained from SD rat embryos showed that CXCL1 specifically induced neurogenesis of dopaminergic neurons (Edman et al., 2008). Another study using SD rats found that the production of CXCL1 by neurons and endothelial cells preceded, and was correlated with, neutrophil infiltration into the brain (Johnson et al., 2011). The receptors for CXCL1, CXCR1 and CXCR2, were also present in the brains of SD rats at two weeks and two months of age (Danik et al., 2003.) CXCR1 was present on a significant number of neurons, and approximately half of the neurons sampled expressed both CXCR1 and CXCR2 (Danik et al., 2003). The importance of CXCL1 for neurodevelopment and the presence of CXCR1 and CXCR2 in the rat brain strongly suggest that the effects of polyI:C treatment will be altered by CXCR1/CXCR2 antagonism.

In the present experiments, the CXCR1/CXCR2 antagonist CXCL8(3–72)K11R/G31P (G31P) was administered to antagonize the receptors of CXCL1. G31P is a synthetic, mutated form of human IL-8 that binds CXCR1 and CXCR2 with high

affinity (Zhao et al., 2009). Previous rodent work with a guinea pig model of airway endotoxemia found that neutrophil infiltration and activation within the airways was blocked (>95%) by G31P administration (Zhao et al., 2009). G31P also reduced neutrophil infiltration into the airways and production of the cytokines CXCL8, IL-1 and TNF in guinea pigs treated with *Klebsiella pneumoniae* (Wei et al., 2013). Thus, our hypothesis was that treating pregnant dams with G31P before and after polyI:C would prevent the effects of elevated CXCL1.

### **Behavioral Tests of Cognition**

The primary method used to assess treatment outcomes in the present experiments was behavioral testing of the offspring, with tests chosen to assess processes that are typically impaired in human schizophrenia. The major symptom domains of schizophrenia are positive, negative and cognitive. Research has shown that cognitive symptom levels are strong predictors of patient functioning (Green, 2006; Kitchen et al., 2012) and, yet, cognitive functioning is not improved by antipsychotic treatment (Wallace et al., 2011). To address this problem, the United States National Institute of Mental Health formed the Measurement and Treatment Research to Improve Cognition in Schizophrenia (MATRICS) initiative (Marder and Fenton, 2004). The MATRICS initiative identified seven cognitive domains that are typically impaired in schizophrenia patients, which are attention/vigilance, working memory, reasoning and problem solving, processing speed, visual learning and memory, verbal learning and memory and social cognition. Based on this information, Young et al. (2009) sought to identify the most valid rodent behavioral tests to form a preclinical cognitive test battery for schizophrenia.

PPI was identified by Young et al. (2009) as a useful test of sensorimotor gating, a component of attention that occurs early in stimulus processing. In healthy humans and other animals, the presentation of a non-startling acoustic tone, called a prepulse, prior to a louder pulse reduces the startle reflex to that pulse. In schizophrenia patients, inhibition of the startle reflex by a non-startling prepulse is typically decreased (Braff et al., 1992; Weike et al., 2000). These PPI impairments are typically attributed to widespread dysfunction involving the striatum, hippocampus, thalamus, frontal and parietal regions of the brain (Takahashi et al., 2011). PPI is an especially useful measure for clinical testing, as an identical test can be delivered to humans and rodents (Swerdlow et al., 1994).

Several researchers have examined PPI in the polyI:C model. PPI impairments were reported at three months of age in SD offspring of dams injected with polyI:C (4.0 mg/kg, i.v.) on gestational day (GD) 15 (Wolff and Bilkey, 2008, 2010). Using the same treatment protocol, PPI impairments were also shown in Wistar rats tested on PND 90-98 (Klein et al., 2013; Mattei et al., 2014) and in Long-Evans (LE) rats tested on PND 56-57 (Howland et al., 2012). Similar findings have been obtained in C57BL/6J mice, who showed PPI impairments after prenatal exposure to polyI:C (20 mg/kg, i.p.) on embryonic day (ED) 12.5 (Smith et al. 2007). In contrast to these findings, Fortier et al. (2007) found that treatment of SD dams with polyI:C (two consecutive daily doses of 750–1000 µg/kg, i.p.) starting on ED 10, ED 15 or ED 18 had no significant effect on PPI in the offspring. These findings suggest that the species and strain chosen, the polyI:C treatment protocol and the timing of testing in the offspring can all impact performance on the PPI test.

As noted above, impaired visual learning and memory was also identified as a cognitive symptom of schizophrenia by the MATRICS initiative (Marder and Fenton, 2004). Human patients have shown impairments in multiple forms of visual learning and memory including spatial and non-spatial, recognition and recall, and short- and long-term memory (Heinrichs and Zakzanis, 1998). Specific impairments have been shown for visual pattern recognition (Cestari et al., 2013), and immediate and delayed recall and recognition of complex figures (Bozikas et al., 2006). To investigate visual learning and memory in rodents, Young et al. (2009) recommended the novel object recognition test, which relies upon animals' innate preference for exploring novel stimuli to demonstrate memory (Ennaceur and Delacour, 1988). In the test, an animal first explores two identical copies of an object. Then, following a delay, the animal explores an additional copy of the original object and a novel object. Memory is inferred when the animal spends more time exploring the novel object. Findings from several studies indicate that the perirhinal cortex (PRh) is the most important cortical area for novel object recognition memory performance (Winters et al., 2008).

A more complex, but related form of recognition memory is associative recognition memory. A study of patients with first episode schizophreniform psychosis and established schizophrenia showed that both patient groups were impaired on multiple tests of visuospatial memory compared to control subjects (Wood et al., 2002). However, results from the same study showed that only patients with established schizophrenia were impaired on the visuospatial paired-associate learning test. This pattern of results caused the researchers to conclude that visuospatial associative memory impairments emerge as patients transition from first-episode schizophreniform psychosis to

established schizophrenia (Wood et al., 2002). Similar findings were obtained in a functional magnetic resonance imaging (fMRI) study, in which schizophrenia patients showed similar performance to controls on a visual recognition test, but were impaired on the visual associative recognition test (Montoya et al., 2007). fMRI images showed that schizophrenia patients had less prefrontal cortex (PFC) activity during the visual associative recognition test than controls, suggesting that diminished PFC activity was responsible for their impairment (Montoya et al., 2007). This fMRI finding coincides with a large body of research demonstrating PFC hypoactivity in schizophrenia (da Silva Alves et al., 2008).

One commonly used test of associative recognition memory in rodents is the object-in-place (OIP) recognition memory test (Bussey et al., 2000). In this test, an animal first explores four distinct objects in a square arena. Then, following a delay, the animal explores identical copies of the same four objects with the location of two of the objects switched. Memory for an object-location association is inferred when the animal spends more times exploring the objects that have been moved (novel object-location association) than it spends exploring those that have not been moved. A lesion study demonstrated that OIP test performance is dependent on processing in both the PRh and the medial prefrontal cortex (mPFC) of rats (Barker et al., 2007).

Howland et al. (2012) administered both the novel object recognition test and the OIP test to polyI:C-exposed LE rats. They found that the offspring of polyI:C-treated dams showed intact novel object recognition, but were impaired on the OIP test. This finding corresponds with human data showing greater impairments on associative recognition tests than simple visual recognition tests (Wood et al., 2002; Montoya et al.,

2007). Based on this result, and the comment from Young et al. (2009) that multiple tests are appropriate for studying visual learning and memory in rodents, the present experiments included the OIP test and the visual component of the crossmodal recognition test (described below) to assess visual recognition memory.

The final cognitive domain examined in the present experiments was multisensory integration, a rapidly growing subject of research in the schizophrenia field.

Multisensory integration is the formation of a distinct representation of a stimulus that incorporates information from multiple sensory modalities. Accumulating evidence suggests that schizophrenia patients are impaired in this process, although findings have been mixed. Schizophrenia patients showed less reaction time facilitation to multisensory targets than controls (Williams et al., 2010), suggesting that the processing of multimodal stimuli is impaired. However, electroencephalography studies have obtained contradictory findings, with one group finding impairments in audiovisual integration (Stekelenburg et al., 2013), and the other finding enhancements (Stone et al., 2011).

The most appropriate rodent test of multisensory integration is the crossmodal recognition memory test (Winters and Reid, 2010). This test includes visual, tactile and crossmodal components. Visual and tactile testing require the animal to form a memory of an object in one sensory modality (unimodal) and, subsequently, to recognize that object in the same modality. Crossmodal testing requires the animal to recognize an object visually based on a previously acquired tactile representation. Bilateral lesions of the PRh, the posterior parietal cortex (PPC) or unilateral lesions of PRh and PPC in opposite hemispheres were administered to LE rats to investigate the cortical areas

responsible for test performance (Winters and Reid, 2010). PRh lesions impaired memory on the visual and crossmodal tests, while PPC lesions impaired performance on the tactile and crossmodal tests. Most significantly, animals with crossed unilateral PRh/PPC lesions were selectively impaired on the crossmodal test, suggesting that multisensory integration is dependent on an association between these cortical areas in rats. Further studies showed that hippocampal lesions have no effect on crossmodal recognition memory (Reid et al., 2012), while the PFC appears to contribute (Reid et al., 2013). To the best of the author's knowledge, no published research has examined the crossmodal recognition memory of animals exposed prenatally to polyI:C, or any other inflammatory treatment.

## **Hypotheses**

Based upon the findings outlined above, several hypotheses were formed for the present experiments. PolyI:C treatment was expected to trigger an acute inflammatory event that increased the production of numerous cytokines in maternal serum and lung tissue. Elevations were specifically predicted for IL-1 $\beta$  and IL-6, based on findings in mice (Meyer et al., 2006), and IL-10 and TNF- $\alpha$ , based on findings from mice and rats (Meyer et al., 2006; Song et al., 2011). Maternal concentrations of the chemokine CXCL1, the rodent analogue of human IL-8, were also expected to increase following polyI:C treatment. This hypothesis was formed based on the human association between prenatal exposure to elevated maternal IL-8 and the development of schizophrenia in adult offspring (Brown et al., 2004b). Although there is no published data showing the effects of G31P in rats, rats treated with the combination of polyI:C and G31P were expected to show decreased serum cytokine concentrations compared to rats who were

only treated with polyI:C. The finding that G31P treatment decreased IL-1 and TNF production in guinea pigs (Wei et al., 2013) supports this hypothesis, although it is possible that species differences would change the response of rats to G31P.

For the PPI test of sensorimotor gating, maternal polyI:C treatment was hypothesized to decrease the PPI of the offspring. This finding would correspond with previous data published by our laboratory (Howland et al., 2012), as well as numerous other studies examining PPI in the polyI:C model (Smith et al., 2007; Wolff and Bilkey, 2008, 2010; Klein et al., 2013; Mattei et al., 2014). The group of offspring whose mothers were treated with the combination of polyI:C and G31P were expected to show increased PPI compared to those whose mothers were only treated with polyI:C.

Maternal polyI:C treatment was also hypothesized to impair associative recognition memory performance of the offspring on the OIP test. This hypothesis was also based on previously published work from our laboratory (Howland et al., 2012). For the crossmodal test of multisensory integration, all treatment groups were expected to show similar memory abilities on the visual and tactile recognition tests. These tests only require animals to use one sensory modality to form memories of, and recognize, objects. These relatively simple requirements, as well as the finding that the offspring of polyI:C-treated rats did not show impaired novel object recognition memory (Howland et al., 2012) strongly suggest that polyI:C would not cause impairments on the visual or tactile recognition tests. For the crossmodal test, the offspring of polyI:C-treated dams were expected to show recognition impairments. There is no previously published data showing the effects of polyI:C treatment on crossmodal recognition memory. However, the cognitively complex nature of the test, as well as the involvement of the PFC in



performance (Reid et al., 2013) strongly suggest that polyI:C-exposed offspring would be impaired. G31P treatment was expected to reverse all polyI:C-induced recognition memory impairments, due to its effects on the inflammatory response.

## **METHODS**

### **Animals**

Timed pregnant LE dams (Charles River Laboratories, Quebec, Canada) arrived at the laboratory on GD 7. They were singly housed with food (Purina Rat Chow) and water available ad libitum in a colony room (maintained at 21°C) with a 12 h light/dark cycle (lights on at 0700 h). Data was collected from four separate squads of rats. Experiments were conducted during the light phase and experimenters were blind to the treatments during testing. All experiments were performed in accordance with the Canadian Council on Animal Care and were approved by the University of Saskatchewan Animal Research Ethics Board.

### **Maternal Treatment**

On GD 15, dam weight and rectal temperature (Homeothermic Blanket System, Harvard Instruments, MA, USA) were recorded. Dams were anesthetized for approximately 10 min using isoflurane (5% induction and 2.5% maintenance) and given one i.v. tail vein injection of either saline or polyI:C (4.0 mg/kg, high molecular weight; InVivoGen, San Diego, CA, USA). Half of the dams from each treatment group also received three i.p. injections of G31P (500 µg/kg; 1 h before, 48 h after, and 96 h after polyI:C or saline treatment) (Gordon et al., 2005; Zhao et al., 2009). Other than injections, weight, and temperature recordings (8, 24 and 48 h after treatment) dams were

undisturbed until PND 1, when litters were weighed and culled to a maximum of ten pups (six males where possible). On PND 21 litters were weaned into same-sex sibling cages and male pups were randomly selected for behavioral testing. Care was taken to control for litter effects in behavioral testing, as 1) no greater than six males from a litter were included in one treatment group and 2) all treatment groups included rats from ten or more distinct litters.

### **Multiplex Assays and ELISAs for Cytokines**

Dams were deeply anesthetized with isoflurane 3 h after polyI:C or saline administration. The rats were decapitated and maternal lung tissue was rapidly dissected and flash frozen using liquid nitrogen. Trunk blood samples were allowed to clot for 60 min (room temperature) and then centrifuged at 12,000 rpm for 4 min. Serum was pipetted from each sample and flash frozen. All samples were stored at -80°C until analysis. Bio-Plex Pro Assay multiplex kits were used to quantify protein in the samples, as has been done previously (Garay et al. 2013). Levels of IL-1 $\beta$ , IL-6, TNF- $\alpha$ , CXCL1, IFN $\gamma$ , IL-1 $\alpha$ , IL-2, IL-4 and IL-10 were first quantified in tissue samples from rats treated with either saline ( $n=3$ ) or polyI:C ( $n=5$ ). A subsequent experiment measured cytokines in samples from rats treated with either saline-saline (sal-sal), saline-polyI:C (sal-polyI:C), G31P-saline (G31P-sal), or G31P-polyI:C ( $n=4$  for each group). Note that G31P was only given 60 min before polyI:C in this experiment. ELISAs were performed on maternal serum for CXCL1 (GRO $\alpha$ /KC) and CXCL2 (GRO $\beta$ /MIP-2; R&D Systems, Inc., Minneapolis, MN) to confirm the measurements from the multiplex assays for CXCL1 and to measure CXCL2. Bio-Plex and ELISA assays were carried out by a separate research group (laboratory of Dr. John Gordon, Department of Medicine,

University of Saskatchewan) with all procedures following the manufacturer's instructions.

### **Behavioral Testing**

Behavioral tests were conducted according to published protocols (Winters and Reid, 2010; Howland et al., 2012; Jacklin et al., 2012). Testing occurred in the following order: PPI during puberty (PND 35-36) and young adulthood (PND 56-57), followed by recognition memory testing (PND 60-80).

#### *PPI*

Two SR-LAB startle boxes (San Diego Instruments, San Diego, CA, USA) were used. Prior to each session, a rat was placed into a cylindrical enclosure within the startle box. The enclosure uses a 12 bit resolution motion sensor to quantify movement. Each session had a constant background noise (70 dB) and began with a 5 min acclimatization, followed by six pulse-alone trials (120 dB, 40 ms). Pulse-alone (6), prepulse + pulse (72) and no stimulus (6) trials were then presented in a pseudorandom order, followed by 6 additional pulse-alone trials. Prepulse + pulse trials began with a 20 ms prepulse of 3, 6, or 12 dB above background (70 dB). Prepulse-pulse intervals were 30, 50, 80 or 140 ms between the onset of the prepulse and the onset of the 120 dB pulse. The inter-trial interval varied randomly from 3 to 14 seconds. The boxes were cleaned with 40% ethanol between sessions.

#### *Object-in-Place Recognition Memory*

The testing apparatus was a white open-field arena (60 X 60 X 60 cm) with one black wall, constructed from corrugated plastic (Figure 4A). Three 10 min habituation sessions, in which a rat is placed into an empty arena, occurred prior to testing. During

the first two sessions, two rats were simultaneously habituated in the same room, in separate arenas. In the third session, rats were individually habituated. One day after the third habituation, rats explored four distinct objects for 5 min (sample phase). Following a 1 h delay in which the rats returned to their home cages, rats explored identical copies of the same four objects with the location of two of the objects switched (test phase).

#### *Crossmodal Recognition Memory*

The testing apparatus was a Y-shaped arena (constructed of corrugated plastic), with one entrance arm and two object arms (10 X 27 cm) (Figure 4C, E, G). Testing included three distinct components: the visual, tactile and crossmodal memory tests. Transparent plastic barriers were inserted in front of the objects during visual, but not tactile phases. One red light bulb (60 W) was illuminated during the tactile phases, preventing the rats from seeing the objects, but allowing video recordings to be made of the rats' behavior. One paired and one individual habituation session (10 min) occurred prior to testing. White overhead lighting and the red light bulb were separately illuminated for half of each habituation, with the order of illumination counterbalanced. Testing began one day after the second habituation. The order of administration of the visual, tactile and crossmodal tests was counterbalanced. All tests included a 3 min sample phase and 2 min test phase separated by a 1 h delay, in which rats were returned to their home cages, between phases. The maze contained two identical copies of an object during the sample phase, and a third copy of the original object with a novel object during the test phase.

## Data Analysis

All results are reported as group means  $\pm$  the standard error of the mean (SEM). Values greater than 2 standard deviations above or below the mean were considered outliers, and were removed prior to statistical analysis. For PPI, outlier criteria was determined using mean PPI on all long-interval (50, 80, 140 ms) trials. SPSS Statistics version 22 (IBM) was used to conduct all statistical tests, using a significance value of  $p < 0.05$ . Non-significant findings are reported as n.s. Two way analysis of variance (ANOVA) with polyI:C and G31P treatment as between-subjects factors were predominantly used for analysis. Corrections were made for violations of sphericity using Mauchly's Test, where appropriate. Post hoc analyses were performed separately using t-tests.

### *PPI*

PPI was calculated by averaging the startle amplitudes for each trial type, and the percent PPI for each prepulse intensity was calculated using the formula:  $[100 - (100 \times \text{startle amplitude on prepulse} + \text{pulse trials}) / (\text{startle amplitude on pulse-alone trials})]$  (Howland et al., 2004a, 2004b, 2012).

### *Recognition Memory*

Exploration was scored when a rat was judged to be actively exploring an object with its nose directed within 2 cm of the object and its head or vibrissae moving, but not when it was standing on top of the object or not directing attention towards it. A discrimination ratios (DR), calculated as the time spent exploring (novel-familiar)/(novel + familiar), was used to quantify memory (Czakoff and Howland, 2011; Howland et al., 2012).

## RESULTS

### Effects of polyI:C and G31P on cytokine concentrations in maternal serum and lung

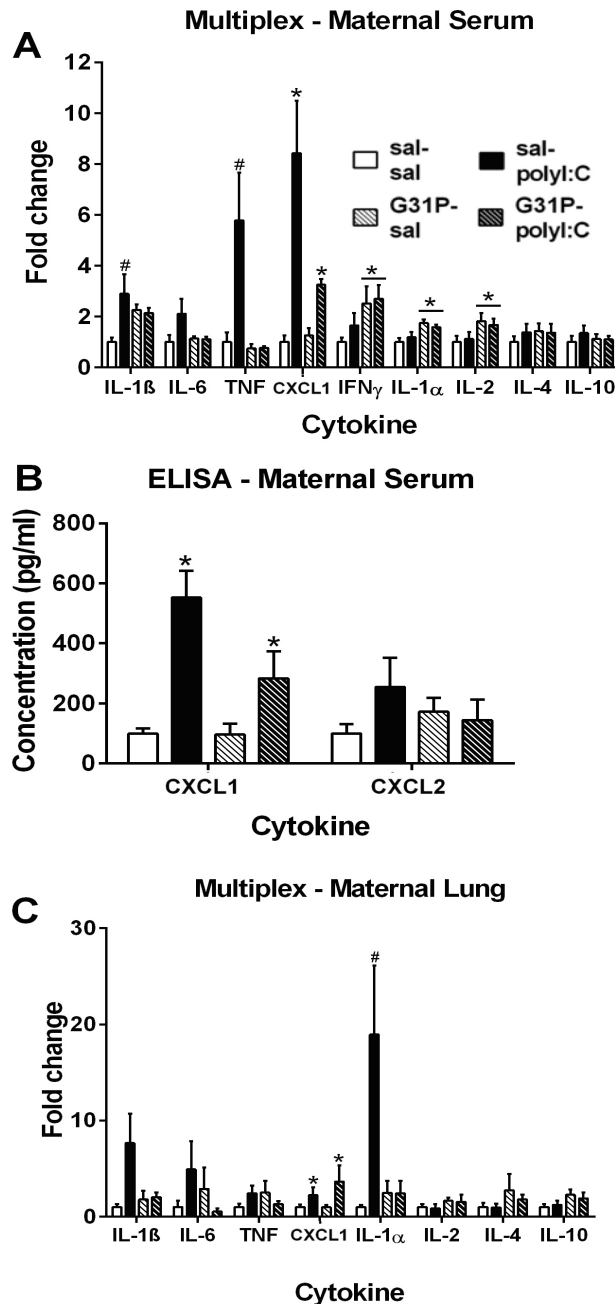
#### *Maternal Serum (Figure 1A, B)*

Analysis of maternal serum samples demonstrated that the treatments significantly increased the concentration of a number of cytokines. An ANOVA revealed a significant main effect of polyI:C treatment for CXCL1 ( $F(1,20) = 7.61, p = 0.012$ ) and significant main effects of G31P for IFN $\gamma$  ( $F(1,20) = 6.30, p = 0.021$ ), IL-1 $\alpha$  ( $F(1,20) = 7.12, p = 0.015$ ) and IL-2 ( $F(1,20) = 5.19, p = 0.034$ ). While no significant interactions were observed, inspection of the data revealed that polyI:C increased the concentration of other proinflammatory cytokines including IL-1 $\beta$ , IL-6, and TNF- $\alpha$ . Analysis of the simple main effects of polyI:C treatment, excluding rats treated with G31P from analysis, revealed significant increases for IL-1 $\beta$  ( $t(14) = -2.16, p = 0.049$ ) and TNF- $\alpha$  ( $t(14) = -2.22, p = 0.044$ ).

To further characterize the chemokine changes in maternal serum 3 h after polyI:C and G31P treatment, ELISAs for CXCL1 (GRO $\alpha$ /KC) and CXCL2 (GRO $\beta$ /MIP-2) were performed (Figure 1B). The results indicated that polyI:C treatment significantly increased the concentrations of CXCL1 ( $F(1,18) = 20.25, p < 0.001$ ) without altering the concentration of CXCL2 (n.s.). While there was no main effect of G31P treatment for either chemokine (n.s.), the interaction between polyI:C and G31P was close to significant for CXCL1 ( $F(1,18) = 3.48, p = 0.079$ ) but not CXCL2 (n.s.). Inspection of the data revealed that G31P treatment had a tendency to reduce levels of CXCL1 in serum from polyI:C-treated dams.

*Maternal Lung (Figure 1C)*

The assay failed to detect IFN $\gamma$  in 14/22 samples so results are not reported. Analysis of the data with a two-way ANOVA revealed that polyI:C treatment significantly increased CXCL1 concentration in the lung ( $F(1,17) = 5.00, p = 0.039$ ). G31P did not significantly increase concentrations of the cytokines, although the effect on IL-10 levels was close to significant ( $F(1,17) = 4.07, p = 0.060$ ). Large effects were also noted for IL-1 $\beta$  and IL-1 $\alpha$  following polyI:C treatment, although these differences were not significant when either the main effects or interaction terms were considered. Analysis of simple main effects for saline and polyI:C treatment revealed a significant effect of polyI:C treatment for IL-1 $\alpha$  ( $t(11) = -3.0, p = 0.042$ ) but not IL-1 $\beta$  (n.s.).



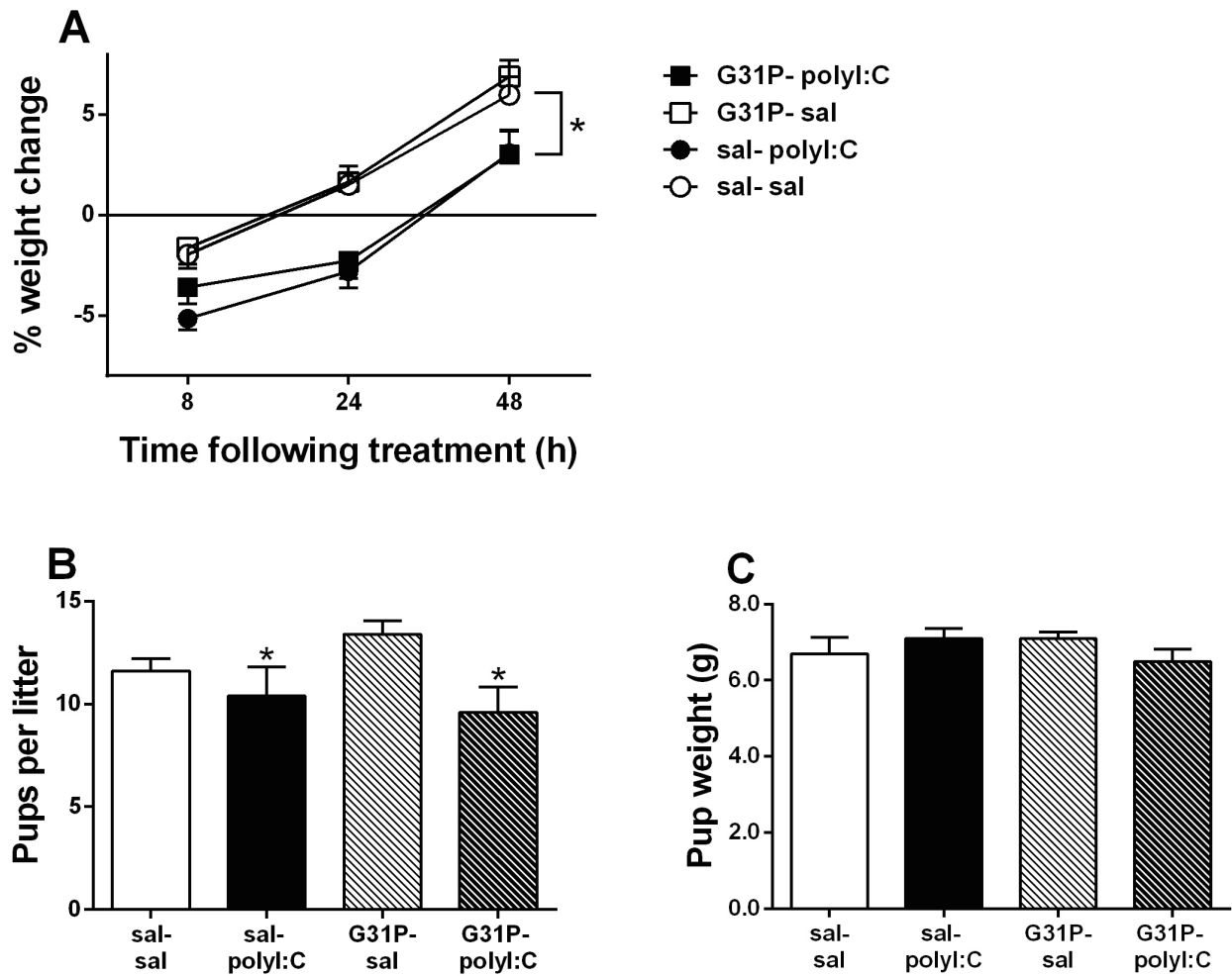
**Figure 1. Effects of polyI:C and G31P treatment on cytokine concentrations in maternal serum (A, B) and maternal lung (C).** A. PolyI:C treatment significantly increased serum concentrations of CXCL1, IL-1 $\beta$ , TNF- $\alpha$ . G31P treatment significantly increased serum levels of IFN $\gamma$ , IL-1 $\alpha$ , and IL-2. Group sizes were: sal-sal ( $n=7$ ), sal-polyI:C ( $n=9$ ), G31P-sal ( $n=4$ ), and G31P-polyI:C ( $n=4$ ). B. Serum concentration of CXCL1, but not CXCL2, was significantly increased by polyI:C treatment. Samples were the same as those in panel A except for that two were not available for testing from the sal-polyI:C group. C. PolyI:C treatment significantly increased concentrations of CXCL1 and IL-1 $\alpha$  in maternal lung tissue. G31P did not have significant effects on any cytokine examined. Tissues were collected 3 h after polyI:C treatment on GD15. Bio-Plex data are presented as fold-change relative to the group mean of the sal-sal treated group as the concentrations of the cytokines in the saline treated group varied. \* indicate a significant main effect of either polyI:C or G31P. # indicate a significant difference between the sal-sal and sal-polyI:C groups.



### **Effects of polyI:C and G31P on the pregnant dams and pups**

PolyI:C treatment significantly decreased the weight of the dams for at least the 48 h following treatment (Figure 2A). An ANOVA (polyI:C and G31P as between-subjects factors; percent weight change as a within-subjects factor) confirmed a main effect of polyI:C ( $F(1,45) = 17.49, p < 0.001$ ), and revealed n.s. effects of G31P treatment and the polyI:C by G31P interaction. After treatment, dams exhibited an average response of weight loss at 8 h ( $-3.09 \pm 0.7\%$ ) and weight gain at 48 h ( $4.74 \pm 1.0\%$ ), consistent with a significant main effect of time following treatment ( $F(1.39,63.37) = 475.50, p < 0.001$ ). Neither polyI:C nor G31P significantly altered temperature in the 48 h following treatment (data not shown).

At birth, the number of pups per litter and the total weight of each litter were taken. PolyI:C treatment significantly decreased litter size ( $F(1,48) = 75.85, p = 0.016$ ) from  $12.35 \pm 0.5$  to  $9.96 \pm 0.9$  pups (Figure 2B). G31P treatment did not affect litter size, with n.s. effects of G31P and the polyI:C by G31P interaction. Average pup weights were not affected by either treatment (Figure 2C) with all main effects and interactions n.s.



**Figure 2. Effects of polyI:C and G31P treatment on maternal weight change (A), number of pups per litter (B), and average pup weight at birth (C).** PolyI:C treatment caused significant weight loss in the 48 h following treatment (A) and decreased the number of pups per litter (B), without affecting pup weight (C). Maternal weight change (A) was normalized to the weight of the dams immediately before the initial saline or G31P treatment on gestational day (GD) 15. G31P or saline (500  $\mu$ g/kg, i.p.) treatments occurred on GD 15, 17, and 19. PolyI:C or saline (4 mg/kg, i.v.) treatments were administered on GD 15, 60 min after G31P or saline. Group sizes were sal-sal ( $n=15$  dams), sal-polyI:C ( $n=11$  dams), G31P-sal ( $n=11$  dams), G31P-polyI:C ( $n=12$  dams). B, C. Number of litters assessed for pups per litter and average pup weight were: sal-sal ( $n=15$ ), sal-polyI:C ( $n=11$ ), G31P-sal ( $n=11$ ), G31P-polyI:C ( $n=12$ ). \* in panels A and B denote a significant main effect of polyI:C treatment. No significant effects of G31P were observed.

## **Effects of exposure to polyI:C and G31P on PPI in the offspring**

### *PND 35. Startle amplitude (Figure 3A)*

The average startle amplitudes during blocks of pulse-alone trials (before, during and after prepulse + pulse trials) were analyzed to assess habituation during a session. Results of an ANOVA (polyI:C and G31P as between-subjects factors; pulse-alone block as a within-subjects factor) showed a significant effect of block ( $F(1.40, 116.29) = 91.65$ ,  $p < 0.001$ ), as startle was significantly higher during the first block of pulse-alone trials than during the other blocks. All other main effects and interactions were n.s.

### *PND 35. PPI: 30 ms prepulse-pulse interval (Figure 3C)*

As described previously, prepulse facilitation was observed for trials with a 30 ms interval (Howland et al., 2012) and they were analyzed separately from the other trials. An ANOVA (polyI:C and G31P as between-subjects factors; prepulse intensity as a within-subjects factor) revealed a significant effect of prepulse intensity ( $F(2, 166) = 44.95$ ,  $p < 0.001$ ), as rats showed prepulse facilitation at the 3 dB prepulse and PPI at the 12 dB prepulse (data not shown). There was a significant main effect of G31P treatment ( $F(1, 83) = 4.49$ ,  $p = 0.037$ ), with G31P-exposed rats showing less facilitation than those not exposed to G31P. The main effect of polyI:C and the polyI:C by G31P interaction were n.s.

### *PND 35. PPI: 50, 80, 140 ms prepulse-pulse intervals (Figure 3E, G)*

Data for the remaining prepulse-pulse intervals was analyzed using ANOVA (polyI:C and G31P as between-subjects factors; prepulse-pulse interval and prepulse intensity as within-subjects factors). Regardless of treatment, rats exhibited decreased PPI at the 50 ms interval compared to the 80 and 140 ms intervals (Figure 3E), giving a main

effect of prepulse-pulse interval ( $F(1.86,154.75) = 19.77, p < 0.001$ ). A significant effect emerged for prepulse intensity ( $F(2,166) = 373.95, p < 0.001$ ), as rats exhibited higher PPI at higher dB prepulses (Figure 3G). The prepulse-pulse interval by prepulse intensity interaction ( $F(4,332) = 8.22, p < 0.001$ ) and the three-way interaction between prepulse-pulse interval, prepulse intensity and polyI:C treatment ( $F(4,332) = 3.94, p = 0.004$ ) were both significant. G31P treatment did not significantly increase PPI as it did on the 30 ms prepulse-pulse interval trials, although the main effect was close to significant ( $F(1,83) = 3.41, p = 0.068$ ). All other main effects and interactions were n.s.

*PND 56. Startle amplitude (Figure 3B)*

Analysis of startle amplitude during the blocks of pulse-alone trials revealed a significant main effect of block ( $F(1.09,91.49) = 41.57, p < 0.001$ ), as startle was higher on the first block of pulse-alone trials than the other pulse-alone trial blocks. All other main effects and interactions were n.s.

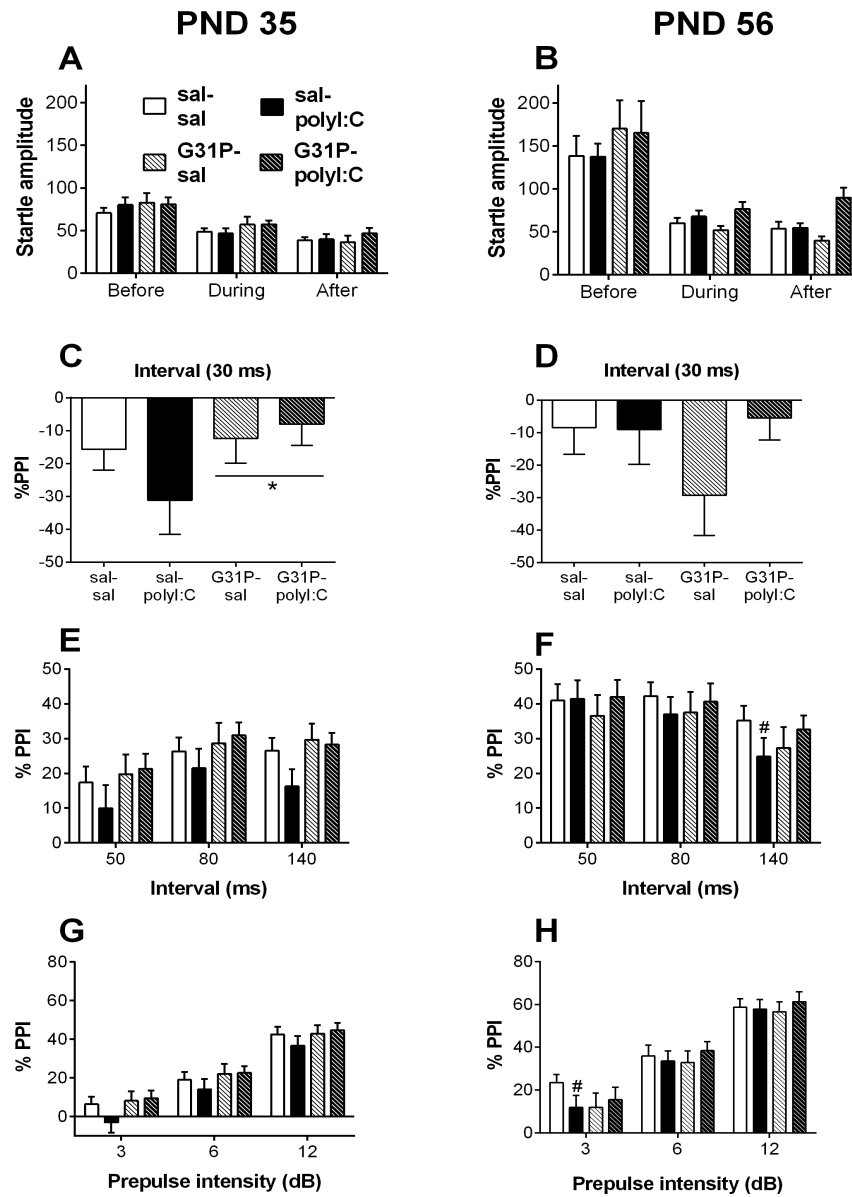
*PND 56. PPI: 30 ms prepulse-pulse interval (Figure 3D)*

Similar to the results from PND 35, a significant effect of prepulse intensity was found ( $F(2,168) = 53.92, p < 0.001$ ). However, at this age neither polyI:C or G31P caused significant alterations in PPI when a 30 ms prepulse-pulse interval was tested (n.s.).

*PND 56. PPI: 50, 80, 140 ms prepulse-pulse intervals (Figure 3F, H)*

Data analysis for these trials revealed significant main effects of prepulse-pulse interval ( $F(2,168) = 21.24, p < 0.001$ ) and prepulse intensity ( $F(1.82,152.80) = 213.20, p < 0.001$ ). In contrast to PND 35, at PND 56 the effect of interval was caused by an overall decrease in PPI with increasing interval. All other effects and interactions in this

analysis were n.s. An analysis of simple main effects, excluding animals exposed to G31P, was performed to allow for a comparison with previous findings. The effects of prepulse-pulse interval ( $F(2,90) = 13.98, p < 0.001$ ) and prepulse intensity ( $F(2,90) = 102.26, p < 0.001$ ) were significant, as was seen in the analysis including G31P animals. This simple analysis also revealed a prepulse-pulse interval by polyI:C interaction that was close to significant ( $F(2,90) = 2.79, p = 0.067$ ). A t-test comparing PPI on trials with the longest prepulse-pulse interval (140 ms) revealed a significant difference ( $t(45) = 2.21, p = 0.032$ ) such that sal-polyI:C rats showed decreased PPI compared to sal-sal control rats (Figure 3F). Another t-test comparing PPI on long-interval trials with the 3 dB intensity prepulse revealed a significant difference ( $t(51) = 2.19, p = 0.033$ ) between sal-polyI:C and sal-sal rats (Figure 3H).



**Figure 3. Acoustic startle responses and prepulse inhibition (PPI) of the offspring following maternal polyI:C and G31P treatment.** A, B. Acoustic startle responses (startle amplitude, arbitrary units) for 120 dB pulse trials before, during, and after the PPI trials at postnatal day (PND) 35 (A) and 56 (B) (sal-sal,  $n=27$ ; sal-polyI:C,  $n=21$ ; G31P-sal,  $n=21$ ; G31P-polyI:C,  $n=23$ ). C, D. % PPI for trials with a 30 ms prepulse-pulse interval at PND 35 (C) and 56 (D). Data is averaged for the 3, 6, and 12 dB prepulse intensities. Negative % PPI values reflect an increase in startle to trials with a prepulse. G31P-exposed rats showed less prepulse facilitation than other rats (main effect of G31P). E, F. % PPI averaged by prepulse intensity for the 50, 80, and 140 ms prepulse-pulse intervals at PND 35 (E) and 56 (F). G, H. Percent PPI averaged by prepulse-pulse interval for 3, 6 and 12 dB prepulse intensities at PND 35 (G) and 56 (H). Group sizes for C, E, G: sal-sal ( $n=24$ ), sal-polyI:C ( $n=20$ ), G31P-sal ( $n=21$ ), G31P-polyI:C ( $n=22$ ). Group sizes for D, F, H: sal-sal ( $n=26$ ), sal-polyI:C ( $n=21$ ), G31P-sal ( $n=19$ ), G31P-polyI:C ( $n=22$ ). Number of litters included in testing: sal-sal ( $n=15$ ), sal-polyI:C ( $n=11$ ), G31P-sal ( $n=11$ ), G31P-polyI:C ( $n=12$ ). # in panels F and H show the significant decreases in PPI for sal-polyI:C rats compared to sal-sal rats for trials with a 140 ms interval (F) and for trials with a 3 dB prepulse (H).

## Effects of exposure to polyI:C and G31P on recognition memory

### *Exploration Times (Table 1)*

With one exception, all treatment groups explored the objects for similar amounts of time during both the sample and test phases of the memory tests, as two way between-subjects ANOVAs revealed n.s. effects of polyI:C, G31P and polyI:C by G31P. During the sample phase of the crossmodal test, however, polyI:C exposure increased exploration from  $30.63 \pm 1.5$  sec to  $36.40 \pm 2.4$  sec ( $F(1,73) = 4.96, p = 0.029$ ).

### *Object-in-place memory (Figure 4A, B)*

ANOVA analysis of DRs showed that polyI:C-exposed rats were impaired on the OIP test compared to those not exposed to polyI:C, as evidenced by a main effect of polyI:C treatment ( $F(1,64) = 10.16, p = 0.002$ ). G31P treatment did not alter memory performance, as the effects of G31P and polyI:C by G31P were n.s. One sample t-tests comparing the DR to 0, a DR score indicating chance memory performance, showed that sal-sal rats exhibited significant memory ( $t(18) = 6.12, p < 0.001$ ) while sal-polyI:C rats were impaired ( $t(16) = 2.107, p = 0.05$ ). Significant memory was also shown by G31P-sal rats ( $t(14) = 4.035, p = 0.001$ ). Despite the strong performance of G31P-sal rats, G31P-polyI:C rats were severely impaired on the test ( $t(16) = 0.323, p = 0.751$ ).

### *Crossmodal recognition memory (Figure 4C-H)*

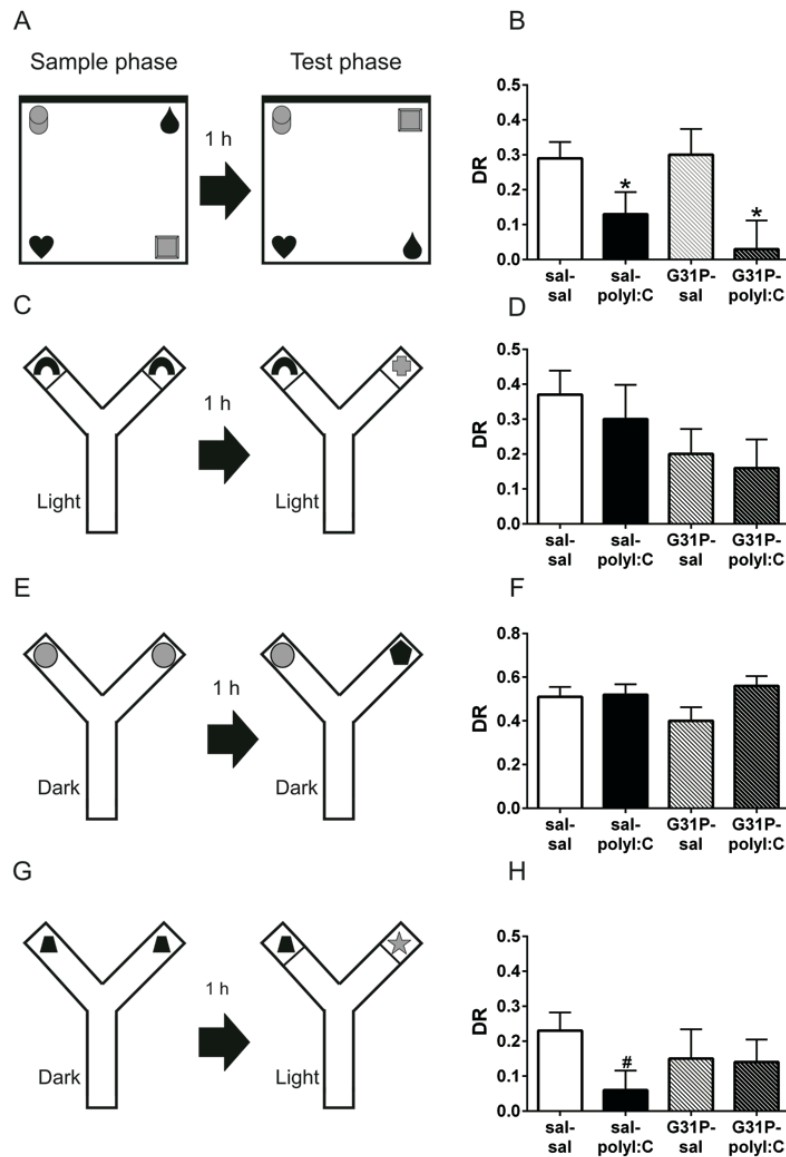
Analysis of DRs on the visual and tactile memory tests showed n.s. main effects of polyI:C, G31P, and the polyI:C by G31P interaction, although the G31P-exposed rats tended to show lower DRs on the visual component of the test (Figure 4D). An analysis using one sample t-tests comparing to a DR of 0 found that G31P-polyI:C rats did not show significant memory compared to chance on the visual test ( $t(12) = 1.929, p =$

0.078). All other groups showed significant memory on both the tactile and visual tests. On the crossmodal portion of the test (Figure 4H), rats were tested for their ability to use information gained from a tactile experience during the sample phase to guide exploration of visual stimuli during the test phase. Sal-sal control rats showed evidence of significant memory ( $t(22) = 4.42, p < 0.001$ ), similar to results described previously (Winters and Reid 2010; Jacklin et al., 2012). Sal-polyI:C rats failed to show significant memory as reflected by a mean DR not different from chance ( $t(19) = 1.06, p = 0.30$ ). G31P-saline rats did not show significant memory on the test ( $t(18) = 2.03, p = 0.057$ ), while G31P-polyI:C rats did show significant memory ( $t(18) = 2.18, p = 0.043$ ). ANOVA failed to reveal significant main effects of polyI:C, G31P or a significant polyI:C by G31P interaction. However, a separate analysis of the simple main effect of polyI:C treatment showed that sal-polyI:C rats were significantly impaired on the crossmodal test compared to sal-sal rats ( $t(41) = 2.22, p = 0.032$ ).



**Table 1. Total exploration time of the objects (s  $\pm$  SEM) during the sample and test phases of the object-in-place (OIP), visual, tactile and crossmodal recognition memory tests.** The total time for the sample phase is presented, whereas the time for the first min is presented for the test phase. No significant differences in exploration were present, except for the increased exploration caused by polyI:C treatment during the sample phase of the crossmodal recognition test. \* indicates the significant main effect of polyI:C treatment.

<b><u>Test</u></b>	<b><u>Phase</u></b>	<b><u>Group</u></b>	<b><u>Exploration (s)</u></b>
<b>OIP</b>	Sample	sal-sal	102.21 $\pm$ 2.1
		sal-polyI:C	105.20 $\pm$ 2.7
		G31P-sal	105.68 $\pm$ 3.7
		G31P-polyI:C	97.67 $\pm$ 3.1
	Test	sal-sal	20.03 $\pm$ 0.9
		sal-polyI:C	22.52 $\pm$ 1.4
		G31P-sal	21.08 $\pm$ 1.6
		G31P-polyI:C	22.78 $\pm$ 1.5
<b>Visual</b>	Sample	sal-sal	3.84 $\pm$ 0.4
		sal-polyI:C	4.27 $\pm$ 0.3
		G31P-sal	3.94 $\pm$ 0.3
		G31P-polyI:C	4.20 $\pm$ 0.5
	Test	sal-sal	2.34 $\pm$ 0.3
		sal-polyI:C	1.98 $\pm$ 0.2
		G31P-sal	1.92 $\pm$ 0.2
		G31P-polyI:C	2.53 $\pm$ 0.4
<b>Tactile</b>	Sample	sal-sal	39.33 $\pm$ 2.6
		sal-polyI:C	36.47 $\pm$ 1.8
		G31P-sal	38.50 $\pm$ 3.1
		G31P-polyI:C	34.90 $\pm$ 1.9
	Test	sal-sal	15.19 $\pm$ 0.9
		sal-polyI:C	14.82 $\pm$ 1.0
		G31P-sal	14.65 $\pm$ 1.1
		G31P-polyI:C	14.99 $\pm$ 1.1
<b>Crossmodal</b>	Sample	sal-sal	31.36 $\pm$ 2.0
		sal-polyI:C	35.86 $\pm$ 3.5*
		G31P-sal	29.60 $\pm$ 2.2
		G31P-polyI:C	36.91 $\pm$ 3.2*
	Test	sal-sal	3.44 $\pm$ 0.3
		sal-polyI:C	2.71 $\pm$ 0.2
		G31P-sal	3.48 $\pm$ 0.3
		G31P-polyI:C	3.60 $\pm$ 0.5



**Figure 4. Associative (A, B), visual (C, D), tactile (E, F), and crossmodal (G, H) recognition memory of the offspring following maternal polyI:C and G31P treatment.** Schematics using an overhead view of each test are shown in panels A, C, E and G. A 1 h delay was used between the sample and test phases of all tests. Discrimination ratios (DR) by group are depicted in panels B, D, F, H; note: the y-axis varies among these panels. B. Rats from the polyI:C groups had significantly decreased associative memory, as assayed by the object-in-place test (sal-sal,  $n=19$ ; sal-polyI:C,  $n=17$ ; G31P-sal,  $n=15$ ; G31P-polyI:C,  $n=17$ ). Rats in the sal-polyI:C group also had significantly lower crossmodal memory (H). Group sizes for D: sal-sal,  $n=18$ ; sal-polyI:C,  $n=14$ ; G31P-sal,  $n=19$ ; G31P-polyI:C,  $n=13$ . Group sizes for F: sal-sal,  $n=22$ ; sal-polyI:C,  $n=20$ ; G31P-sal,  $n=19$ ; G31P-polyI:C,  $n=21$ . Group sizes for H: sal-sal,  $n=23$ ; sal-polyI:C,  $n=20$ ; G31P-sal,  $n=17$ ; G31P-polyI:C,  $n=19$ . Number of litters included in B: sal-sal ( $n=12$ ), sal-polyI:C ( $n=11$ ), G31P-sal ( $n=11$ ), G31P-polyI:C ( $n=12$ ). Number of litters included in D,F,H: sal-sal ( $n=15$ ), sal-polyI:C ( $n=11$ ), G31P-sal ( $n=11$ ), G31P-polyI:C ( $n=10$ ). \* represent main effects, # represent simple main effects comparing sal-sal and sal-polyI:C rats.

## DISCUSSION

The results of the experiments outlined above provide insight into how MIA and chemokine receptor antagonism during pregnancy affect the cognitive abilities of rat offspring. The most notable findings included: 1) elevated levels of the chemokine CXCL1 3 h after polyI:C treatment in maternal serum and lung tissue, 2) replication of the polyI:C-induced OIP recognition memory deficit, as published in Howland et al. (2012), and 3) the first evidence of a polyI:C-induced impairment on the crossmodal recognition test of multisensory integration. Overall, the behavioral effects of polyI:C treatment were smaller than those obtained with previous batches of LE rats in our laboratory, some of which were published in Howland et al. (2012). G31P treatment also had less significant effects than anticipated, as there were no significant reversals of the behavioral impairments caused by exposure to polyI:C. There are multiple explanations that can be considered for the findings obtained in these experiments.

### **Cytokine elevations following treatment with polyI:C and G31P**

To the best of the author's knowledge, there have not been any publications using rats to investigate the effects of polyI:C treatment on levels of the chemokine CXCL1 in maternal serum and lung tissue. The observation of an acute CXCL1 elevation provides construct validity for polyI:C treatment as an animal model of schizophrenia, as elevations of the analogous human chemokine IL-8 in maternal serum have been associated with the development of schizophrenia in adult offspring (Brown et al., 2004b) as well as the structural brain alterations typically seen in the disorder (Ellman et al., 2010).

When simple main effects that excluded G31P-treated dams from analysis were calculated, polyI:C treatment also caused significant increases in serum levels of the cytokines TNF- $\alpha$  and IL-1 $\beta$ . TNF- $\alpha$  increases correspond with results from a human study, which showed that elevated maternal serum concentrations of TNF- $\alpha$  were associated with the development of a variety of major psychotic disorders (Buka et al., 2001). In contrast, elevated levels of IL-1 $\beta$  were not detected in human studies examining associations between maternal cytokines during pregnancy and the development of schizophrenia in adult offspring (Buka et al., 2001; Brown et al., 2004b). Findings from animal research differ from this human IL-1 $\beta$  data, as several rodent studies have shown maternal serum elevations in IL-1 $\beta$  and TNF- $\alpha$  following polyI:C treatment (Meyer et al., 2006; Smith et al., 2007; Song et al., 2011; Arrode-Brusés and Brusés, 2012). This discrepancy for IL-1 $\beta$  could represent a species difference in the inflammatory response of humans and rodents, making it difficult to interpret the meaning of these rodent elevations.

One unanticipated finding of the present experiments was the lack of an IL-6 elevation following polyI:C treatment. An IL-6 increase was predicted based on a detailed study using the polyI:C model in mice. Smith et al. (2007) demonstrated that a single injection of IL-6 into pregnant C57BL/6J mice caused impairments in PPI and latent inhibition of the offspring. They went on to administer an anti-IL-6 antibody at the same time as polyI:C treatment, finding that this co-administration reversed the behavioral impairments. Lastly, Smith et al. (2007) administered polyI:C to IL-6 knockout mice, and found that many of the offspring's behavioral impairments were reduced. Other studies conducted in separate labs using the same strain of mice have also

found that polyI:C treatment increased the concentration of IL-6 in maternal serum (Meyer et al., 2006; Arrode-Brusés and Brusés, 2012). Collectively, these findings provide strong evidence that the effects of polyI:C are at least partially dependent on IL-6 signaling.

There are several possible explanations as to why IL-6 was not significantly elevated in the present experiments. In these experiments, Bio-Plex Pro Assay multiplex kits were used to quantify cytokine levels in maternal serum and lung tissue. This method was chosen because it allows for the quantification of multiple cytokines from a single sample, and it gives comparable measurements to ELISA assays (Eishai and McCoy, 2006). ELISA assays were also used to verify elevations of CXCL1, and to examine levels of CXCL2. It is possible that the measurement techniques employed led to these discrepant results for IL-6. However, the studies that found IL-6 elevations in mice utilized a variety of techniques including ELISA assays (Smith et al., 2007), multiplexed bead-based immunoassay Milliplex Maps (Arrode-Brusés and Brusés, 2012) and Fluorokine MAP mouse kits (Meyer et al., 2006), making it unlikely that the IL-6 elevation was an artifact.

A more plausible explanation for the IL-6 discrepancy is inherent differences in the innate immune systems of C57BL/6J mice and LE rats. While no previously published research has compared the cytokine responses of these animals to polyI:C, there have been studies comparing the behavior of different strains of mice following MIA. In one study, the offspring of NMRI and C57BL/6 mice showed markedly different behavioral profiles following prenatal exposure to lipopolysaccharide (LPS) on GD 17 (Babri et al., 2014). Another study using BTBR and C57 mice showed substantial

behavioral differences in the offspring following exposure to polyI:C, as well as differences in cytokine release by splenocytes *in vitro* (Schwartz et al., 2013). Data showing that adult male SD rats did not show elevated plasma IL-6 4 h after polyI:C treatment provides additional evidence that rats may show different IL-6 responses than mice (Fortier et al., 2004). Further research is clearly needed to characterize the inflammatory and behavioral responses of different species and strains to polyI:C in order to produce more consistent results in the field.

Another area that should be addressed in future research is the measurement of cytokines in maternal and fetal tissues. Attempts were made by our laboratory to quantify cytokines in placenta, brain tissue of the mother, and brain and lung tissue of the fetus using Bio-Plex Pro Assay multiplex kits. Unfortunately, these assays were not sensitive enough to detect the small amounts of cytokines present in many of the samples, despite being designed for small-volume applications (Eishai and McCoy, 2006). Future investigations could benefit from the use of bioassays or total cytokine immunoassays that are able to detect levels of both free and bound cytokines (Malone et al., 2001; Hillyer and Woodward, 2003). Quantifying the levels of cytokines that have not previously been measured after polyI:C treatment would also benefit future studies. Particular attention should be paid to the chemokine CXCL12, which binds the chemokine receptors CXCR4 and CXCR7 in the brain and influences the glutamatergic and GABAergic systems (Guyon, 2014). Quantifying a large number of cytokines from a wider range of tissues in LE rats will increase our understanding of which tissues have the highest cytokine activity, and how these elevations contribute to neurodevelopmental changes in the offspring.

### **Behavioral effects of exposure to polyI:C and G31P**

The behavioral data from the present experiments included several replications of previous results, as well as some novel findings. For PPI testing, the baseline performance of the control animals was comparable with previously published data (Howland et al., 2004a, 2012). At both PND 35 and 56 all groups showed normal habituation, as their startle to 120 dB pulses decreased across the session. Animals also exhibited prepulse facilitation on trials with the shortest prepulse-pulse interval (30 ms), which is a replication of the novel finding published in Howland et al. (2012). On the remaining trial types, saline-exposed rats also exhibited PPI that was comparable to previous findings.

The effects of polyI:C on PPI were smaller than those published in Howland et al. (2012), as no main effects emerged for treatment. Fortier et al. (2007) reported similar insignificant results in a study using polyI:C treatment in SD rats. However, simplified data analysis comparing the PPI of sal-sal rats with sal-polyI:C rats on trials with the longest prepulse-pulse interval (140 ms), and the 3 dB prepulse intensity did reveal significant polyI:C-induced impairments. These reduced effects of polyI:C treatment on PPI are difficult to explain, considering that the effects seen on the OIP test are very similar to previous results (Howland et al., 2012). One possible explanation could be that the PPI impairments did not emerge until after PND 56, as the other rat studies that have found PPI impairments following polyI:C treatment administered testing around three months of age (Wolff and Bilkey, 2008, 2010; Klein et al., 2013; Mattei et al., 2014). A

delayed emergence of PPI impairments would correspond with the reduced treatment effects of polyI:C in these experiments.

The only significant effect that emerged for G31P treatment on PPI testing was reduced prepulse facilitation on trials with the 30 ms prepulse-pulse interval during adolescence (PND 35). The mechanisms underlying prepulse facilitation at short intervals have not been elucidated, making it unclear how G31P treatment caused this effect. However, this finding indicates that G31P exposure altered cognitive functioning on PND 35. If further behavioral studies using G31P are conducted, more tests should be administered at this age to look for additional differences.

PolyI:C exposure caused significant associative memory impairments on the OIP test, as evidenced by a main effect of polyI:C treatment. Comparison of the animals' DRs to chance performance ( $DR=0$ ) showed that sal-sal rats demonstrated significant memory, while sal-polyI:C rats did not. These results serve as a useful replication of published findings (Howland et al., 2012) that validate the use of the polyI:C model for studying the associative recognition memory impairments seen in schizophrenia. This polyI:C-induced impairment was not reversed by G31P treatment, as G31P-polyI:C rats did not show significant memory compared to chance. It seems unlikely that G31P treatment caused the impairment seen in this group, as G31P-sal rats showed significant memory on the OIP test.

PolyI:C treatment also selectively impaired multisensory integration, as sal-polyI:C rats showed significant memory on the visual and tactile tests, but were impaired on the crossmodal test. Sal-sal rats showed significant memory compared to chance on all tests, with similar DRs to those seen in several papers (Winters and Reid, 2010; Reid



et al., 2012, 2013). This pattern of results did not lead to a main effect of polyI:C treatment for the crossmodal test, because G31P-polyI:C rats exhibited significant crossmodal recognition memory. Despite this insignificant main effect, these findings provide evidence that the polyI:C model is useful for studying multisensory integration in the context of schizophrenia. Replication of these findings using polyI:C or another model of MIA would provide further strength for this claim.

The performance of G31P-exposed animals on the crossmodal tests was very different from the predicted results. While there were no significant treatment main effects or interactions on the visual and tactile tests, G31P-exposed animals showed a trend toward impaired visual recognition memory, with G31P-polyI:C rats failing to show significant memory on the visual test. G31P-exposed rats also showed a trend toward decreased crossmodal recognition memory, with G31P-sal rats showing impairments on the crossmodal test. Both of these results could be explained by visual impairments in G31P-exposed rats, as the test phase of the crossmodal test is a purely visual assessment.

### **Considerations for effects of G31P treatment**

Maternal treatment with G31P during gestation could induce visual impairments in the offspring by altering optic development of the fetus, or development of the cortical areas involved in visual processing. As this was the first documented investigation of visual learning and memory following maternal G31P administration, this impairment would have been missed in other studies (Gordon et al., 2005; Zhao et al., 2009; Wei et al., 2013). Further research is needed to determine if G31P treatment does interfere with

development of the visual system, or if other factors were responsible for the G31P-induced visual memory impairments.

Another issue surrounding G31P that deserves further consideration is its ability to bind CXCR1 and CXCR2 in LE rats. G31P not antagonizing CXCR1/CXCR2 effectively could explain the results of the OIP test, in which G31P-sal rats showed significant memory but G31P-polyI:C rats did not. However, other results showed that G31P had significant effects on the dams and their offspring. For example, G31P treatment caused significant serum elevations of the cytokines IFN $\gamma$ , IL-1 $\alpha$  and IL-2, all of which were unanticipated findings. G31P also significantly altered the PPI exhibited by the offspring during adolescence. These effects of G31P may have occurred through the intended mechanism of CXCR1/CXCR2 antagonism, or through other unidentified processes.

### **Targeted vs. Broad-spectrum Approaches**

A larger issue than the efficacy of G31P as a CXC chemokine receptor antagonist is whether or not inhibiting the activity of one chemokine will ever be sufficient to prevent the impairments caused by polyI:C, or the development of human schizophrenia. Independent human studies have found different cytokines, including TNF- $\alpha$  (Buka et al., 2001) and IL-8 (Brown et al., 2004b) to be associated with the development of schizophrenia. These discrepant findings strongly suggest that multiple cytokines are involved in the development of the disorder. Moreover, these studies do not provide us with complete information about how those particular cytokine elevations affect other components of the inflammatory system. Cytokine interactions are extremely complex,

and the compensatory mechanisms that take place when the activity of one cytokine is inhibited are beyond the scope of our current understanding.

The utility of more generalized treatment approaches that inhibit the action of multiple pro-inflammatory processes is starting to gain attention. The antibiotic minocycline improved symptoms in rats whose mothers were treated with polyI:C (Mattei et al., 2014) and in human patients in early-phase schizophrenia (Levkovitz et al., 2010). A downside to these positive findings is that the side effects of such broad-spectrum treatments could potentially be high. Continuing to increase our understanding of the specific inflammatory processes underlying neurodevelopmental disorders will allow for the creation of more targeted anti-inflammatory drugs in the years to come.

#### **Future considerations for animal models**

This future research will not be possible without some experiments using animal models. The polyI:C model is valuable, largely because of its high construct validity. In spite of its strengths, one weakness of the model that is often overlooked is the inherent assumption that all offspring who are exposed to MIA will go on to develop schizophrenia. It is very clear from human research that this claim is not accurate (Brown et al., 2004a, 2004b). Increased use of double-hit animal models that include a genetic predisposition for the disorder and an environmental trigger, such as MIA, will bring the animal data closer in line with human findings. A double-hit model of polyI:C treatment in dominant negative- disrupted in schizophrenia 1 (DN-DISC1) transgenic mice has already been conducted, although the researchers did not compare the behavior of polyI:C-exposed wild-type mice with polyI:C-exposed DN-DISC1 mice to quantify the effects of this genetic mutation (Nagai et al., 2011).

## **General conclusions**

In conclusion, the present experiments quantified the effects of maternal treatment with the viral mimetic polyI:C and the chemokine receptor antagonist G31P on the cognitive functioning of LE rat offspring. PolyI:C treatment triggered a significant acute inflammatory event, which included an elevation of the chemokine CXCL1 in maternal serum and lung tissue. As CXCL1 is considered the rodent analogue of human IL-8, and maternal IL-8 elevations during pregnancy are associated with the development of schizophrenia in adult offspring (Brown et al., 2004b), this finding increases the construct validity of the polyI:C model. The offspring of polyI:C-treated rats exhibited impaired associative recognition memory on the OIP test, replicating a previously published finding (Howland et al., 2012). These rats also exhibited impaired multisensory integration on the crossmodal test, which is a novel finding for the polyI:C model. The behavioral effects of G31P were inconsistent across tests, and G31P treatment never successfully reversed a polyI:C-induced impairment. Given our current understanding of the etiology of schizophrenia, broad spectrums anti-inflammatory drugs should continue to be investigated as adjuvant therapies for the disorder. Future research incorporating the use of double-hit animal models will, hopefully, foster the identification of more targeted anti-inflammatory drugs that are able to treat the cognitive symptoms of schizophrenia.

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