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The Effects of Two Novel Anti-Inflammatory Compounds On Prepulse Inhibition and Neural Microglia Cell Activation in a Rodent Model of Schizophrenia

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by

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ABSTRACT

The Effects of Two Novel Anti-Inflammatory
Compounds On Prepulse Inhibition and Neural Microglia
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Recent studies have shown elevated neuroinflammation in a large subset of individuals diagnosed with schizophrenia. A pro-inflammatory cytokine, tumor necrosis factor-alpha (TNFα), has been directly linked to this neuroinflammation. This study examined the effects of two TNFα modulators (PD2024 and PD340) produced by our collaborators at P2D Bioscience, Inc., to alleviate auditory sensorimotor gating deficits and reduce microglial cell activation present in the polyinosinic:polycytidylic (Poly I:C) rodent model of schizophrenia. Auditory sensorimotor gating was assessed using prepulse inhibition and microglial activation was examined and quantified using immunohistochemistry and confocal microscopy, respectively. Both PD2024 and PD340 alleviated auditory sensorimotor gating deficits and reduced microglia activation and thereby demonstrated the ability to treat both the behavioral and neuroinflammatory aspects of the disorder. These results are significant and suggest that neural TNFα is a potential pharmacological target for the treatment of schizophrenia.

DEDICATION

I dedicate this thesis to my parents. I have never known a time in my life where they haven't supported or encouraged me. Their constant influence both inside and outside of the classroom has been central to my achievements. Thank you both for all you have done and continue to do for me. I promise to continue becoming the best version of myself in all that I undertake.

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CHAPTER 1

INTRODUCTION

Schizophrenia (SCZ) is a chronic and debilitating neurobehavioral disorder that affects an estimated 21 million people worldwide (World Health Organization 2018). SCZ negatively affects cognition, emotion, and behavior. The age at onset of SCZ is classically observed in adolescence or during the beginning of adulthood. The prevalence of SCZ and other related psychotic disorders range between 0.25% and 0.64% in the United States (Kessler et al. 2005; Wu et al. 2006; Desai et al. 2013; National Institute of Mental Health 2018). Medications and other therapeutic expenses for the treatment of SCZ annually costs the U.S. an estimated $62 billion (Fleischhacker et al. 2014). Diagnosis is not based on laboratory testing, but instead arises from clinical observation and self-reporting (Gejman et al. 2010). Currently, there is no cure for treating the behavioral disorder. Therefore, it becomes increasingly important to investigate new approaches in order to understand SCZ and the psychophysiology involved.

Schizophrenia: Symptoms, Psychophysiology, & Treatments

Schizophrenia is a severe mental disorder accompanied by three categories of symptoms: positive, negative, and cognitive. Associated positive symptoms include delusions, hallucinations (usually auditory), paranoia, unusual ways of thinking, and movement disorders. These symptoms are deemed positive due to the psychotic behaviors present that are not observed in normal individuals. Positive symptoms are "added" to common behavior. Negative symptoms relate to changes in common behavior and emotion and include a flat affect, reduction in speech, anhedonia, and difficulties in beginning and finishing activities. Negative symptoms are

"removed" from common behavior. Cognitive symptoms relate to disturbances in memory and thinking. These symptoms are comprised of a lack of concentration, impaired executive functioning, and the loss of ability to apply information shortly after just learning it. There are many associated risk factors for developing SCZ, but the reason for this development is not yet fully understood. The main risk factors shown to contribute to SCZ development later in life include early viral infection, brain lesions, prenatal and neonatal malnutrition, recreational drug abuse, and influences that impact psychosocial functioning (National Institute of Mental Health 2018). SCZ and associated symptoms are hypothesized to stem from a variety of biochemical factors including increased dopamine D2 receptor sensitivity, glutamate dysregulation, and recently, neuroinflammation.

Dopamine D2 Receptor Supersensitivity

There are five dopamine receptors (D1, D2, D3, D4, D5) found throughout the CNS. They are G-protein-coupled receptors (GPCRs) located within the nerve cell membrane that are activated by dopamine binding. The central hallmark of SCZ continues to be hypersensitivity of the dopamine D2 family of receptors (Howes et al. 2017). Support for this notion comes from positive symptom alleviation when treated with drugs that block dopamine binding to D2 receptors (Kusumi et al. 2015). Dopamine is a catecholamine neurotransmitter involved in reward, executive function, motor control, motivation, and arousal. Dopamine is synthesized from its precursor molecule, L-DOPA, which can also be used to make norepinephrine and epinephrine. Discovery of the D2 receptor came from analysis of where antipsychotic drugs bind via radioligand labeling (Madras 2013). Dopamine receptor signaling dysfunction has also been associated with many other psychiatric disorders. It has been hypothesized that dopamine D2

receptor supersensitivity observed in those diagnosed serves as compensation for initial trauma, which thereby increases dopamine neurotransmission, overstimulates the dopamine system, and creates SCZ symptomatology (Seeman and Seeman 2014). It is worth note that cocaine and amphetamine hijack the dopaminergic system to induce delusional paranoia that resembles some of the positive symptoms of SCZ. Dopamine D2 receptor supersensitivity has been shown to lead to delusions (Howes and Kapur 2009), hallucinations (Garrett and Silva 2003), and cognitive deficits (Seeman and Seeman 2014). Neonatal treatment with quinpirole, a dopamine D2/D3 agonist, increases the sensitivity of the dopamine D2 receptor and is used as an animal model of SCZ (Brown et al. 2012).

In SCZ, there is not a change in the number of dopamine D2 receptors, but rather overactivity and increased signaling of dopamine. Treatment today relies on drugs that block dopamine D2 receptors with some affinity, although newer atypical antipsychotics also focus on signaling in the serotonergic, histaminergic, and noradrenergic neurotransmitter systems. The quality of life for those diagnosed has significantly improved as a result of antipsychotic treatment regimes, however complete normalization of behavior is rarely accomplished (Harvey et al. 2012). Antipsychotics with better efficacy are still being developed today as a result of our constantly increasing knowledge regarding the pathology of neurotransmission in SCZ.

Glutamate & NMDA Receptor Hypothesis of SCZ

Stone et al. (2007) proposed a differing hypothesis suggesting SCZ may arise from a deficit in glutamatergic neurotransmission, particularly through dysfunction of the N-methyl-D-aspartate (NMDA) receptor. Glutamate is the most abundant excitatory neurotransmitter in the nervous system (Meldrum 2000) and is involved in learning and memory processes (McEntee

and Crook 1993). Glutamate binds to different receptors, one of which is the NMDA receptor. The NMDA receptor is a ligand-gated ion channel located on the membrane of nerve cells that is activated upon glutamate binding. NMDA receptors are also important in memory and synaptic plasticity (Li and Tsien 2009). Binding of glutamate to the NMDA receptor allows Ca^{2+} to enter into the neuron. Excess intracellular Ca^{2+} ultimately damages neurons, suggesting a mechanism for neurodegeneration (Harvey et al. 2012). Multiple studies (Krystal et al. 1994; Morgan and Curran 2006; Javitt 2007) have implicated the role of NMDA receptor dysfunction in SCZ, resulting from administration of non-competitive NMDA receptor antagonists (ketamine and its analog, phencyclidine). Phencyclidine (PCP) and ketamine inhibit cell depolarization to induce SCZ-like associated deficits (Johnson and Jones 1990). Long-term administration of PCP in rats was shown to elicit negative symptoms (Egerton et al. 2008) by blocking the reuptake of dopamine, serotonin, and norepinephrine (Harvey et al. 2012), whereas ketamine generated cognitive deficits. Evidence for NMDA receptor dysfunction and glutamate hypofunction has also been observed in post mortem brain tissue (Howes et al. 2015). In SCZ, glutamate receptor localization has been shown to be abnormally regulated, which could be due to dysregulation of glutamate receptor trafficking molecules (Funk et al. 2009). Functional changes to the NMDA receptor also impact glutamatergic signaling, as a subunit (GRIN2A) of the NMDA receptor was found to be associated with SCZ in genetic analyses (Ripke et al. 2014).

Evidence for NMDA receptor dysfunction and glutamate dysregulation has also been observed *in vivo*, using Single-Photon Emission Computerized Tomography (SPECT) and Proton Magnetic Resonance Spectrometry (1H-MRS). Using SPECT, Pilowsky et al. (2006) showed unmedicated patients diagnosed with SCZ had left hippocampal NMDA receptor activity deficiencies. Bustillo et al. (2014) found an elevated glutamine:glutamate ratio in the anterior

cingulate cortex using 1H-MRS, with a strong correlation between increased glutamine:glutamate ratio and psychosis. Untreated, but diagnosed individuals have been shown to have increased glutamate levels in the caudate nucleus, a region of the brain responsible for many motor processes (de la Fuente-Sandoval et al. 2011), thereby further strengthening the NMDA receptor/glutamate hypothesis. It is also worth note that dopamine antagonizes the glutamate system, thus reducing glutamate release. Dopamine and glutamate dysregulation must both be considered when analyzing mechanisms behind the development of SCZ. Thus, it can be hypothesized that dopamine supersensitivity and deficits in glutamate neurotransmission are both major biochemical factors that contribute to the disorder.

History of Treatment & Discovery of Antipsychotics

Treatment for SCZ before the middle of the 20th century initiated by confining patients indefinitely to psychiatric hospitals, commonly referred to as mental asylums (Luo 2015). Therapeutic pharmacological medicine did not yet exist, which lead to unusual approaches. Patients diagnosed with SCZ were subjected to a plethora of interventions no longer used today, including barbiturate-induced sleep therapy, comas (from high insulin doses), and neurosurgeries (Valenstein 1986). Barbiturate-induced sleep therapy was proposed to alleviate lethargy in those diagnosed, thus improving patient psychosocial functioning. However, this therapeutic technique only acutely improved symptomatology, as patients would revert back to their initial state once the barbiturate's effect was eliminated (López-Muñoz et al. 2005). Early studies suggested high insulin doses could reduce psychotic episodes, but instead resulted in loss of consciousness and seizures. Lobotomy of the frontal lobe was also suggested as a method to reduce agitation and impulsive behavior, but was shown to lead to cognitive impairment with no reported benefits

(Lavretsky 2008). Psychoanalysis, an investigation into the subconscious factors that contribute to mental illness, was used in both the U.S. and the United Kingdom, but proved ineffective (Guttmacher 1964; Lavretsky 2008). As a result, the quality of life for those diagnosed did not significantly improve and patients were without an effective treatment option.

The first antipsychotic drug, chlorpromazine, was discovered in 1952. Since the discovery of chlorpromazine, approximately fifty antipsychotic drugs have been developed. Chlorpromazine was created by the modification of promethazine, a drug used to treat circulatory shock after surgery (Lavretsky 2008). Chlorpromazine was found to alleviate many positive symptoms of SCZ, increase social functioning, and reduce relapse rates (Lavretsky 2008; Luo 2015). The discovery of chlorpromazine thus began the development of first-generation antipsychotics (FGAs). Common FGAs still prescribed today include chlorprothixene, perphenazine, and haloperidol. FGAs are competitive dopamine D2 receptor antagonists, but also modulate other neurotransmitters, as discussed with SGAs. FGA are termed either as "low-potency" or "high-potency" to indicate their affinity for the D2 receptor. By blocking the dopamine D2 receptor, FGAs reduce psychosis and delay symptom exacerbations (Carpenter and Koenig 2008). However, FGA are generally not effective for treating negative symptoms and generate many unwanted side effects. For example, reserpine was discovered shortly after chlorpromazine and was already used to treat hypertension. Reserpine reduces synaptic dopamine release (Carpenter and Koenig 2008) leading to a decrease in dopamine levels within the brain. As a result of depleting dopamine, treatment with reserpine has been shown to create Parkinson's-like symptoms (Luo 2015), further confirming the existence of negative side-effects that accompany FGA treatment. Second-generation antipsychotics (SGAs) were developed to reduce common side effects generated by FGA treatment and to improve negative

symptomatology. The first SGA, risperidone, was discovered in 1996. Other commonly known SGAs include clozapine, olanzapine, ziprasidone, and quetiapine. SGAs block dopamine D2 receptors in the brain similar to FGAs, and also block cholinergic, adrenergic, serotonergic (particularly 5-HT_{2A}), and histaminergic receptors. Risperidone, in particular, blocks serotonin 5-HT_{2A} receptors with more affinity than dopamine D2 receptors. Quetiapine also blocks D2 receptors with a greater affinity than 5-HT_{2A} receptors (López-Muñoz and Alamo 2013). Another SGA with a different pharmacodynamic profile than risperidone and quetiapine is clozapine. Clozapine is a weak dopamine D2 receptor antagonist, but has a high affinity for other key receptors including 5-HT_2, muscarinic, and α-adrenergic receptors (Chaki et al. 2000). Clozapine and other SGAs have been shown to some extent to ameliorate negative symptoms of SCZ, although none have been shown to be completely effective. Besides serving as an antipsychotic medication, the FDA also approved the use of clozapine as an anti-suicidal treatment option (Meltzer 2005), thereby demonstrating the ability of the drug to be used in multiple capacities. The problems with SGAs have also been due to side effects of these drugs, and their efficacy. The Clinical Antipsychotic Trials for Intervention Effectiveness (CATIE) sponsored by the National Institute of Mental Health revealed that compliance to SGAs was poor, and all were below 20%. Compliance was low primarily due to side effects of these drugs, including most prominently weight gain and extrapyramidal motor effects (Manschreck and Boshes 2007). Therefore, it is clear that new pharmacological targets are greatly needed for treatment of this population.

Problems with Current Treatment

Even though antipsychotic medications remain as the first-line of treatment for SCZ and SCZ-like disorders, many problems also result from current treatments. It is also of note that approximately 20% of patients diagnosed with SCZ do not respond to antipsychotic treatment (Harvey et al. 2012). There are many debilitating side effects that accompany an antipsychotic treatment regime. These include extrapyramidal side effects (EPS), weight gain, and increased risk of diabetes mellitus (DM) development, hyperlipidemia, and agranulocytosis. These side effects have been observed in both typical and atypical antipsychotic treatment regimes, usually on a dose-dependent basis. Henceforth, the focus will be on the accompanying side effects observed in both typical (FGA) and atypical (SGA) antipsychotics, including specific side effects reported more often with certain drugs.

A recent review by Divac et al. (2014) provided a thorough investigation into the EPS that follows treatment with an antipsychotic. EPS are serious and incapacitating, often requiring additional pharmacological intervention. EPS are developed as a result from the blocking of dopamine receptors in the nigrostriatal pathway (Harvey et al. 2012). Acute EPS develop at treatment initiation or when required dosage is increased to achieve therapeutic value. Prolonged treatment with antipsychotics, particularly FGA, can also lead to late-onset EPS, including tardive dyskinesia (involuntary somatic movement). EPS manifestations impair motor patterns and include acute dystonia, akathisia, and Parkinsonism.

Acute EPS usually responds to a reduction in antipsychotic medication, often attracting patients to discontinue treatment in order to alleviate the symptoms. Within the first few days of antipsychotic treatment, approximately 50% of patients treated with haloperidol developed acute EPS (Divac et al. 2014). Discontinuation of treatment also results from the risk for developing

tardive dyskinesia (TD), which severely decreases quality of life and can even persist after the abolishment of treatment (Casey 2004; Rosenheck 2007). The average prevalence of TD after treatment has been shown to be between 24-30% (Casey 1999; Llorca et al. 2002). Clozapine, an atypical antipsychotic, is the only known antipsychotic that does not cause acute EPS, but does cause agranulocytosis, a life-threatening condition of severe leukopenia that if untreated, can lead to septicemia. Clozapine can also suppress the bone marrow and increase the risk of seizures and cardiovascular abnormalities (Harvey et al. 2012).

Acute dystonia is a neurological motor pattern disorder that results in repetitive muscle contractions, which can lead to abnormally fixed postures. Acute dystonia is commonly seen in first-generation antipsychotics (FGA). Although less common, dystonia can still be present in second-generation antipsychotics (SGA). Kamishima et al. (2009) found approximately 7% of patients that were treated with long-acting risperidone developed acute dystonia reactions. Akathisia (approximately 50% of all cases of EPS) is a movement disorder characterized by the inability to remain still due to feelings of inner restlessness. Akathisia is a common side effect that follows treatment, but remains poorly understood. Approximately 25% of patients treated with a FGA medication develop akathisia (Divac et al. 2014). Anticholinergic medication does not affect akathisia, but dose-reduction of antipsychotics or introduction of beta adrenergic blockers and benzodiazepines have proven to be effective (Shirzadi and Ghaemi 2006; Poznic et al. 2012).

Parkinsonism is characterized by tremor, decreased body movement, and rigidity and thereby mimics some of main symptoms seen in Parkinson's disease. Parkinsonism develops within the first few days up to several months after treatment starts. 26% of patients treated with olanzapine and 55% treated with haloperidol develop Parkinsonism (Lieberman et al. 2003).

Parkinsonism induced by antipsychotic medications is reversible via anticholinergics and dose-reduction, although symptom duration varies. However, anticholinergics have to be closely monitored when administered to elderly patients due to the accompanying cognitive decline, urinary retention, and exacerbation of glaucoma that can result (Divac et al. 2014). Late-onset EPS like TD can occur months to years after treatment begins. TD is characterized by involuntary body movements that results from antipsychotic drug treatment. Shirzadi and Ghaemi (2006) found the risk of developing TD to be the highest during the first five years following FGA treatment. Anticholinergic intervention following antipsychotic administration has also been shown to exacerbate existing TD (Divac et al. 2014).

Both FGA and SGA drugs have been found to contribute to weight gain and negative metabolic side effects (Allison et al. 1999; Wirshing et al. 1999; Allison and Casey 2001). Weight gain in particular is mediated by the antihistaminic action of FGA and SGA drugs. Short or long-term administration of antipsychotics antagonizes histamine H1 receptors, affects hypothalamus-brain stem circuitry, and ultimately increases appetite and fat accumulation (He et al. 2013). For example, the common over-the-counter anti-allergy medication, Benadryl (diphenhydramine), also antagonizes histamine receptors and if used long-term, can lead to significant increases in body weight (Hasnain and Viewig 2013). SGA are associated with a higher risk for developing these side effects (Harvey et al. 2012). Allison and Casey (2001) showed mean body weight increases of 4.45 kg (clozapine), 4.15 kg (olanzapine), 2.92 kg (sertindole), 2.10 kg (risperidone), and 0.04 kg (ziprasidone) following treatment. Nemeroff (1997) also found the most commonly used dose of olanzapine (15 mg/day) lead to a 10-kg weight increase during the first year of treatment, with weight gain being dose-dependent. Rondanelli et al. (2006) highlighted the importance of dosage on side effects, showing elderly

patients that received 1.4 mg/day of risperidone or 4.4 mg/day of olanzapine, or 75 mg/day of quetiapine had no change in weight during a one year period.

Antipsychotic intervention may lead to an increased risk for the development of diabetes mellitus (DM). Multiple studies have reported antipsychotic use to be strongly associated with weight gain (e. g. olanzapine, clozapine) and also strongly increase the risk for developing DM and diabetic ketoacidosis (Fuller et al. 2003; Guo et al. 2006; Haupt 2006; Rubio et al. 2006). Multiple studies have also demonstrated increased cholesterol and serum triglyceride levels in patients following treatment with dibenzodiazepine-derived SGA (e. g. clozapine, olanzapine, quetiapine) over a two-month period (Meyer 2002; Wu et al. 2006).

As a result of many unwanted side effects, discontinuation of treatment with both FGA and SGA is often reported. Again, the CATIE study was designed to examine many of the factors involved in discontinuation of antipsychotic medications. Subjects that had received a diagnosis of SCZ were randomly assigned to receive either olanzapine, perphenazine, quetiapine, risperidone, or ziprasidone in a double-blind experimental approach. The subjects were monitored for 18 months or until treatment discontinuation. The CATIE trials found that 74% of participants discontinued their prescribed medication (olanzapine = 64%, perphenazine = 75%, quetiapine = 82%, risperidone = 74%, ziprasidone = 77%) before the 18 months were completed (Swartz et al. 2008). Factors leading to discontinuation included EPS, weight gain, and onset of DM. Weight gain was strongly associated with olanzapine specifically, but was observed in some extent for all medications.

Neuroinflammatory Aspect of Schizophrenia

Inflammation is the initial response of the immune system that results from the host's defense mechanism to eliminate a foreign threat caused by infection or trauma (Chen et al. 2018). Leukocytes migrate to the area of interest and blood supply increases. Early pathogenic infection during gestation has been shown to increase the risk for developing SCZ later in life (Kneeland and Fatemi 2013). Inflammation that follows an early developmental infection persists throughout the lifetime, often causing autoimmune diseases (Ercolini and Miller 2009) and sometimes crosses the blood-brain barrier (BBB) to damage key components within the brain. In turn, neuronal plasticity is disrupted and cytokines/neurotransmitters become abnormally regulated. One hypothesis suggests that disruptions in the developing immune system are driving influences behind psychotic episodes and brain changes (Cannon et al. 2003) observed in SCZ, including enlarged ventricle size, reductions in gray matter and whole-brain volume, and differences in white matter (Vita et al. 2012).

Inflammation is maintained by amplified cytokine production. Cytokines are small glycoproteins involved in cell-to-cell communication between immune cells. Cytokines are produced by many types of cells including, but not limited to, macrophages, lymphocytes, and granulocytes. Cytokines, particularly pro-inflammatory cytokines, contribute to inflammation and remain present until the threat has been neutralized. Pro-inflammatory cytokines are produced primarily by activated macrophages and neurons in the brain to help up-regulate the inflammatory response (Zhang and An 2009).

In those diagnosed with SCZ, there is an unusual and elevated peripheral recruitment pattern (Schroeter et al. 2009) of immune cells, leading to the generation of inflammatory cytokines, particularly tumor necrosis factor-alpha (TNFα) (Miller et al. 2011; McCusker and

Kelley 2013). Post mortem brain studies further confirm this report by demonstrating TNFα levels were significantly (30-50%) higher in the prefrontal cortex (PFC) and hippocampal (HPC) regions of the SCZ brain compared to unaffected individuals (Kim and Webster 2009). TNFα is released from leukocytes (white blood cells) during the acute phase reaction of inflammation and regulates the coordination between immune cells via cellular signaling. Along with generating inflammation, TNFα can induce fever and trigger apoptosis. Elevated TNFα protein levels have already been implicated in autoimmune diseases such as rheumatoid arthritis, where current treatment relies on TNFα inhibitors (e. g. Infliximab, Etanercept, Adalimumab) to reduce inflammation through antagonism of the cytokine (Ma and Xu 2013). TNFα also directly influences the state of microglial cells, which are the residence surveillance cells of the CNS (Nimmerjahn et al. 2005).

Microglia are a type of glial cell found within the brain and spinal cord (Ginhoux et al. 2013) and account for between 5-20% of the total glia population within the CNS parenchyma (Perry 1998). Without a stimulus (e. g. pro-inflammatory cytokine), microglial cells exist in a "M2 state" that is anti-inflammatory and neuroprotective. These microglia in an M2 state help to protect the brain from inflammation and promote neurogenesis and plasticity. When a stimulus, such as increased TNFα protein secretion is present, M2 microglia switch to a "M1 state", which aids in the inflammatory response and becomes neurotoxic to help deal with the threat. Once reactive, microglia cells via a positive feedback loop overexpress pro-inflammatory cytokines and generate reactive oxygen species (ROS) that damages neuronal synapses and leads to unnecessary neuronal apoptosis and necrosis (Howes and McCutcheon 2017). Recent work (Perry et al. 2010; Kettenmann et al. 2011) suggests that dysregulated reactive microglial cells can ultimately lead to disease and pathology development. Reactive microgliosis (increase in

microglial cell number) has also been reported in many CNS diseases in response to elevated inflammation (Ginhoux et al. 2013).

For those diagnosed with SCZ, neuroinflammation is characterized by M1 microglial cell activation. Multiple studies (Steiner et al. 2008; Fillman et al. 2013; Volk 2017) have shown a direct link between microglial activation and SCZ, particularly in brain regions where white matter is present. As a result, it can be suggested that microglial activation is directly related to certain SCZ symptoms. Recent work has demonstrated the effectiveness of anti-inflammatory compounds as treatment options for SCZ. For example, minocycline, a broad-spectrum tetracycline antibiotic that also has anti-inflammatory properties has been shown to significantly reduce positive and negative symptom severity compared to placebo (Solmi et al. 2017; Xiang et al. 2017). Studies also reported that minocycline alleviated prepulse inhibition deficits (Zhu et al. 2016; Giovanoli et al. 2016) in the Poly I:C rodent model of SCZ, which was used for this current study. Minocycline is able to cross the BBB, suggesting potential downregulation of reactive microglial cells, thus reducing neuroinflammation and improving the associated symptomology of SCZ.

P2D Bioscience, Inc. Previous Studies: Focus on Alzheimer's Disease

Neuroinflammation also continues to gain ground as one of the central factors in Alzheimer's disease (AD) pathology. Multiple studies in AD patients (Kwak et al. 2014; Lynch 2014) have demonstrated an elevated recruitment pattern of leukocytes into the brain and their corresponding interactions with resident microglia. It is still unclear if this recruitment brings about damage to the AD brain, but it can be suggested that this increased infiltration is not usually present in unaffected individuals. One of the pro-inflammatory cytokines ultimately

contributing to this recruitment and increased inflammation is tumor necrosis factor-alpha (TNFα), as recent work has shown (McAlpine and Tansey 2008) it to be significantly implicated in the disease. Again, TNFα polarizes microglia into different neuroinflammatory types, often activating them into their M1 state to become pro-inflammatory and neurotoxic. Therefore, our collaborators (Gabbita et al. 2015) investigated the inhibition/modulation of leukocytes upon the interaction with microglial cells through the use of a novel pro-inflammatory cytokine inhibitor, isoindolin-1,3 dithione (IDT), also termed PD2024, to target neural TNFα and determine what effect(s) it had on AD pathology.

One of the drugs used in the present study is PD2024. PD2024 is a small anti-TNF molecule with a molecular weight of 179.0 g/mol and an IC_{50} for TNF-alpha of 3 μM. Gabbita et al. (2015) reported PD2024 was effective for treating many aspects pertaining to AD. In the BV2 microglial cell line, TNFα response from lipopolysaccharide (LPS) stimulation was attenuated alongside of increasing doses of PD2024, thus demonstrating the effectiveness of PD2024 *in vitro*. TNFα attenuation was found to be due to the ability of PD2024 to destabilize TNFα mRNA, thereby decreasing TNFα protein release and secretion.

Then the Gabbita et al. (2015) study investigated the effectiveness of PD2024 to modulate TNFα *in vivo*. Following an IP 5 mg/kg LPS challenge and oral PD2024 treatment, cortical levels of TNFα protein was increased and significantly reduced alongside of increasing concentrations of PD2024. PD2024 was found in the brain (brain coefficient = 0.25) at detectable amounts moderately comparable to levels found in the tissues. These results thereby revealed that PD2024 was orally bioavailable, crossed the blood-brain barrier, and is effective in reducing TNFα protein levels.

Finally, Gabbita et al. (2015) examined the effects of PD2024 on normal body weight and weight gain in the 3xTgAD mouse model of AD. The 3xTgAD model is triple-transgenic and consists of three specific mutations (APP Swedish, MAPT P301L, PSEN1 M146V) used to represent familial Alzheimer's disease. These mutations generate amyloid-beta (Aβ) plaques and neurofibrillary tangles (NFT), which are central pathology observed in those affected. At low doses (10 mg/kg), PD2024 did not affect weight gain in males or females. However, at both medium (25 mg/kg) and high (50 mg/kg) doses, it was found that weight gain was significantly reduced in both genders, but this did not have an effect on health, activity levels, or cognitive abilities.

With direct relevance to AD, the authors reported a dose-dependent response of PD2024 treatment with improved cognitive performance on the Barnes Maze (used to test spatial awareness and long-term memory) in 3xTgAD mice. PD2024 was also found to reduce insoluble amyloid levels and paired-helical filament (PHF) tau, two of the four primary characteristics of human AD. They hypothesized this could be because PD2024 treatment may allow microglia/macrophages to gain an improved ability to alleviate some of the neuropathology involved. PD2024 was also found to increase infiltrating neutrophils in a dose-dependent manner while also reducing TNFα protein in the central nervous system (CNS). This is of particular importance in regard to neuroinflammation because increased TNFα protein levels negatively regulate neutrophils by the suppression of p40, a subunit of interleukin-23 (IL-23). This study found PD2024 upregulates p40 gene expression, thereby suggesting PD2024 decreases neuroinflammation via mediation of increased neutrophil infiltration into the CNS.

The results of this study confirmed a small TNFα modulator such as PD2024 is safe and well-tolerated, decreases neuroinflammation via TNFα mRNA destabilization, allows neutrophil

infiltration into the CNS to clear AD pathology, and improves cognitive performance. PD2024 is also being developed to treat frontotemporal dementia. Since PD2024 was found to modulate neural TNFα, our study aimed to investigate if this could alleviate the associated neuroinflammation observed in a rodent model of SCZ.

A second novel TNFα modulator (PD340) will also be analyzed in this study. PD340 contains an isoindoline backbone and is a structural analog of PD2024. This compound has yet to be tested in a rodent model of any disease. Although the structure of the compound cannot be revealed due to proprietary information, the drug is designed to modulate TNFα in a similar fashion to PD2024 and has a similar pharmacokinetic profile. We thereby expect PD2024 to have a comparable efficacy for alleviating sensorimotor gating deficits and decreasing microglial cell activation.

Poly I:C Rodent Model of Schizophrenia

All experiments conducted in this study use the polyinosinic:polycytidylic acid (Poly I:C) rodent model of schizophrenia. Poly I:C is an immunostimulant that interacts with the rich, positively-charged amino acid surface of the toll-like receptor 3 (TLR3) within the cell to stimulate and activate the innate immune system and trigger the release of a large number of pro-inflammatory cytokines. The activation of pro-inflammatory cytokines by Poly I:C, particularly TNFα, leads to increased microglial cell activation, known to facilitate white matter injury and neuronal apoptosis (Leviton and Gressens 2007; Khwaja and Volpe 2008). Early challenges to the immune system compromise its integrity, thereby disrupting the cytokine equilibrium in the developing brain (Welberg et al. 2000; Seckl 2004; Mueller and Bale 2008). In doing so, Poly I:C generates an infection phenotype that is restricted to a maximum period of 1-2 days through a

surplus of endocrine, autonomic, and behavioral symptoms induced by this immune activation (Cunningham et al. 2007). The corresponding cytokine imbalance disrupts the structural and functional integrity of the developing brain, ultimately leading to long-lasting consequences later in life (Zhao and Schwartz 1998; Patterson 2007; Meyer et al. 2009; Burd et al. 2012).

Past work (Gandhi et al. 2007; Ribeiro et al. 2013) demonstrated neonatal Poly I:C administration in rats elevates pro-inflammatory cytokine levels and reactive microglial cells in the hippocampus (HPC) and prefrontal cortex (PFC) in the brain. As a result, behavioral deficits emerged including cognitive deficits, deficits in sensorimotor gating (Ozawa et al. 2006; Osborne et al. 2017), and anhedonia (Khan et al. 2014; Missault 2014). Also, Poly I:C administered maternally was shown to create dopamine hyperfunction and structural abnormalities in cortical volumes of the HPC and PFC in offspring (Buschert et al. 2016; Meehan et al. 2017). Maternal exposure during gestation (day 9 and 12.5) generated sensorimotor gating deficits, decreased PPI, and increased startle sensitivity (Meyer et al. 2005; Meyer et al. 2006). Therefore, both maternal and neonatal exposure to Poly I:C produce many characteristic symptoms that are hallmarks of SCZ.

Antipsychotic treatment with clozapine (Ribeiro et al. 2013) and risperidone (Piontkewitz et al. 2011) were shown to alleviate many of the behavioral deficits observed in rats that were treated early with Poly I:C, increasing the validity of this model. Deficits in behavior related to brain structure abnormalities have also been found to be reversible following pharmacological treatment, thereby demonstrating predictive validity of the Poly I:C model. The Poly I:C model allows for precise timing of immunogenic impact as well as the intensity of the associated stimulation with defined stages of brain development (Rutledge 1997). This preclinical model thereby represents the ability for the screening of new pharmacological compounds without

regard to drug interactions to be analyzed in both adolescence and adulthood. This is of top priority for the treatment of SCZ due to the onset of clinical symptoms appearing at these two particular time periods.

Our study capitalizes on these principles through the use of neonatal (postnatal days 5-7) Poly I:C treatment in rats to activate the innate immune response early, disrupt the cytokine balance within the brain, generate reactive microglial cells, and result in behavioral deficits that mimic the same behavioral deficits observed in individuals diagnosed with SCZ. In doing so, the Poly I:C model is able to mimic an exposure to an early infection that disrupts neurodevelopment, shown to increase the risk for developing SCZ and SCZ-like disorders later in life (Meyer 2014; Flinkkilä 2016). Using this model, we are able to analyze new pharmacological targets to alleviate associated behavioral and neuroinflammatory aspects of SCZ.

Hypotheses & Rationale

This research seeks to determine the behavioral and neuroinflammatory effects of two novel TNFα modulators produced by our collaborators at P2D Bioscience, Inc. (Cincinnati, OH) in the polyinosinic:polycytidylic (Poly I:C) rodent model of schizophrenia. Using relevant literature and recent work, the following hypotheses were tested: (1) Treatment with Poly I:C will increase TNFα protein levels similar to the neuroinflammatory response for individuals diagnosed with schizophrenia; (2) Novel TNFα modulators will alleviate sensorimotor gating deficits of the rodent Poly I:C model; (3) Novel TNFα modulators will reduce associated neuroinflammation via a decrease in microglial cell activation levels in the hippocampus and prefrontal cortex, two brain areas that mediate sensorimotor gating.

The effects of the two TNF-alpha modulators (PD2024 and PD340) will be assessed through the use of prepulse inhibition and quantification of the prefrontal cortex and hippocampus via immunohistochemistry and confocal microscopy. The results of this study will analyze a novel pharmacological target for schizophrenia. This study will also provide investigation into the neuroinflammatory role of the behavioral disorder through the use of a well-validated rodent model of schizophrenia.

CHAPTER 2

MATERIALS AND METHODS

Experimental Design

Experiment 1 tested the hypothesis that Poly I:C administration increases TNFα protein levels in the brain. In this experiment, pups were intraperitoneally (IP) injected with either Poly I:C (2 mg/kg) or saline (0.9% NaCl) from postnatal days (P) 5-7. Rats were sacrificed at P30 in accordance with when dietary manipulation for the experiments to assess the TNFα modulators was to initiate. The prefrontal cortex (PFC) and hippocampus (HPC) were dissected away and TNFα protein concentration was analyzed using a TNFα enzyme linked immunosorbent assay (ELISA) kit from Biomatik, Inc. (Wilmington, DE).

Experiment 2 tested the hypothesis that novel TNFα modulator PD2024 would alleviate deficits in sensorimotor gating of the rodent Poly I:C model. This was accomplished by equally dividing pups into four groups, each with differing experimental conditions (Poly IC/Control, Poly IC/PD2024, Saline/Control, Saline/PD2024). Pups in the Poly I:C groups (Poly IC/Control, Poly IC/PD2024) were IP injected with Poly I:C (2 mg/kg) from P5-7, whereas pups in the two saline groups (Saline/Control, Saline/PD2024) were IP injected with saline (1 mg/kg) from postnatal days 5-7. All pups were weaned from the female dam at P21 and then subjected to dietary manipulation beginning at P30. Those in the PD2024 groups (Poly IC/PD2024, Saline/PD2024) received a diet containing bioavailable PD2024 and remained on this diet until P67. Those in the control groups (Poly IC/Control, Saline/Control) received a normal diet until P67, where all four groups were then sacrificed. Prepulse inhibition (PPI) was used to assess auditory sensorimotor gating using behavioral software and equipment from Kinder Scientific,

Inc. (Poway, CA). Animals from all groups were behaviorally tested on PPI in both adolescence (postnatal days 44-46) and adulthood (postnatal days 60-67). PPI allowed for behavioral assessment and comparison between the four groups to determine what effect(s) the modulator had on auditory sensorimotor gating.

Experiment 3 tested the hypothesis that the novel TNFα modulator PD340 would alleviate deficits in sensorimotor gating of the rodent Poly I:C model. Again, PD340 is a small anti-TNF molecule that destabilizes TNFα mRNA, thereby decreases TNFα protein formation and later secretion. PD340 contains an isoindoline backbone and is a structural analog of PD2024. Further details regarding PD340 are limited due to proprietary information. Experiment 3 was used to determine basic efficacies of PD340 and if similarities to PD2024 existed. The same procedures were followed as in Experiment 2, with the exception that PD340 was presented in the diet during the adolescent period instead of PD2024.

In both Experiments 2 and 3, we analyzed whether novel TNFα modulators PD2024 and PD340 would reduce the associated neuroinflammation created by Poly I:C administration via a decrease in microglial activation levels. This was accomplished following the same procedures described in the second aim of this study. Upon animal sacrifice at P67, the prefrontal cortex (PFC) and hippocampal (HPC) regions of the brain were dissected away and analyzed via immunohistochemistry (IHC) and confocal microscopy. IHC and confocal microscopy allowed for assessment and comparison of neuroinflammatory levels via microglial cell activation to compare the effects of PD2024 and PD340 across groups.

Subjects

Male Sprague-Dawley rats that were the offspring of adult male and female breeders ordered from Envigo, Inc. (Indianapolis, IN) served as subjects. Female rats were not used as subjects because it has been equivocal as to whether Poly I:C treated females demonstrate PPI deficits (Bitanihirwe et al. 2010; Zhang et al. 2011; Howland et al. 2012). All animals were housed in a climate-controlled vivarium with a 12-hour light/dark cycle throughout the course of this experiment and food and water was available ad libitum. Rats from each litter were randomly assigned to each drug/diet condition. Day of birth was termed as P0. Regardless of experimental condition, animals remained with their respective female dam from postnatal day (P)1-21 and were then socially housed from P22-30. In Experiments 2 and 3, at P30, dietary manipulation began and animals were socially housed until P67, where they were then sacrificed and brain tissue analyses began.

Experiment 1: TNFα Protein ELISA

A total of 17 Sprague-Dawley rats were IP administered saline (1 mg/kg – N=8) or Poly I:C (2 mg/kg – N=9) from postnatal days 5-7 and raised until P30. At P30, brain tissue was harvested and the prefrontal cortex (PFC) and hippocampus (HPC) were dissected away and frozen on dry ice. Brain tissue from these animals were analyzed using a TNFα enzyme-linked immunosorbent assay (ELISA) kit (Biomatik, Wilmington, DE) to verify administration of Poly I:C increased TNFα protein levels in the PFC and HPC.

ELISA Procedure

We utilized a kit from Biomatik, Inc. (Wilmington, DE) to analyze TNFα protein levels. A total of 500 µL of RIPA cell lysis buffer (150 mM NaCl, 50 mM Tris-HCl, 1.0% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulfate) plus protease and phosphatase inhibitors (P5726, P8340, P0044, Sigma-Aldrich, St. Louis, MO) was added to each sample and homogenized using a Fisher Scientific sonic dismembrator 500 (Fisher Scientific, Inc., Atlanta, GA). Homogenates were centrifuged at 10,000g for 5 minutes at 4 °C. The 96-well ELISA plate was pre-coated with anti-TNFα polyclonal antibody (pAb) for each well. The standard curve was prepared using the TNFα standard (10,000 pg/mL) supplied from the manufacturer. The standard was diluted from the supplied diluent provided in the kit to reach a concentration range between 15.6-1,000 pg/mL. Tissue samples were also further diluted (1:50) before the assay. The plate containing the standards and samples were incubated for 1 hour at room temperature. The monoclonal antibody (mAb) was then added to each well, incubated for 1 hour at room temperature, and was then followed by incubation with the conjugate antibody for one hour. Fluorescent visualization was accomplished via the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate to each well for an incubation period of 20 minutes at room temperature, and was then stopped using 2N sulfuric acid. The plate(s) were read immediately following the addition of the stop solution. Optical density was measured using a Bio- Tek ELx 800 microplate reader (Winooski, VT) at a 450-nm wavelength.

Tumor necrosis factor-alpha (TNFα) protein levels were assessed using two TNFα ELISA plates, one for the PFC and the other for the HPC. TNFα levels were analyzed using an independent t-test to compare the effects of drug treatments on TNFα in each brain area. An independent t-test was used to compare the means of the PFC and HPC and was used to

determine significant differences between TNFα protein levels (dependent variable) by drug manipulation (saline or Poly I:C – independent variable).

Experiments 2 & 3: Poly I:C Administration & Dietary Manipulation

In each of Experiments 2 and 3, animals were equally divided into groups. In Experiment 2, those conditions were: Poly IC/Control, Poly IC/PD2024, Saline/Control, Saline/PD2024. In Experiment 3, those conditions were: Poly IC/Control, Poly IC/PD340, Saline/Control, Saline/PD340. In both experiments, rats were intraperitoneally (IP) administered saline (1 mg/kg) or polyinosinic:polycytidylic acid (Poly I:C – 2 mg/kg) from P5-7. This particular period of Poly I:C treatment has been shown to result in auditory sensorimotor gating deficits and in microglial activation in the hippocampus (Ribeiro et al. 2013). At P30, rats were given a normal diet or a diet containing one of the TNFα modulators (Dyets, Inc., Bethlehem PA). For Experiment 2, PD2024 was given in a 10 mg/kg dose, based on past findings (Gabbita et al. 2015) that this dose was effective in a rodent model of Alzheimer's disease. In Experiment 3, PD340 was given at either low (10 mg/kg) or high (30 mg/kg) dose, because it has not been determined which is the more effective dose for this novel drug. The chosen diet (normal or experimental) remained until P67, where the animals were then sacrificed and brain tissue was analyzed.

Experiments 2 & 3: Body Weight & Weight Gain

Animal weights from the four groups (Saline/Control, Poly IC/Control, Saline/PD2024, Poly IC/PD2024) throughout 10-day time intervals (P30, P40, P50, P60) investigating PD2024 are presented in Table 1. Animal weights from the four groups (Saline/Control, Poly IC/Control,

Saline/PD340, Poly IC/PD340) using the same time intervals (P30, P40, P50, P60) as PD2024 are presented in Table 2, which investigated PD340. Average weight and standard errors of the mean are reported. A two-way analysis of variance (ANOVA) was used to determine if there were significant interactions or differences in weights between the groups. The Newman-Keuls post hoc test (p=0.05) was used to analyze significant interactions (p=0.05).

Experiments 2 & 3: Prepulse Inhibition (PPI) Procedure

All animals in Experiments 2 and 3 were tested once daily on auditory sensorimotor gating task as measured through prepulse inhibition (PPI) at two distinct time periods, in adolescence (P44-46) and adulthood (P60-66). Each daily session began with a 5-minute habituation period with only the background noise (70 dB) present. After this habituation was complete, animals were subjected to three different, randomly assigned trial types, which included *pulse*, *prepulse*, and *no stimulus* trials. The *pulse* trial was a 120 decibel (dB) startle pulse administered by itself. The *prepulse* trial was an auditory stimulus that was either 3, 6, or 12 dB above the 70-dB background noise. The *no stimulus* trial was when a stimulus was not provided. A total of 5 *pulse*, 5 *no stimulus*, and 15 *prepulse* trials (5 trials of each 73, 76, and 82 dB) were presented in each training session. Rats were placed in a stainless-steel dome (height = 8 cm) that was attached to a platform (11 cm wide x 15 cm long) mounted on a stainless-steel ellipse in a sound attenuating chamber (28 cm high x 30 cm wide x 36 cm depth). Animal response was recorded and measured (in Newtons) within a 250-millisecond window immediately following stimulus presentation through a computer interface. PPI also has direct translational value for humans and is used today. Electrodes are placed on the orbicularis oculi

and measures acoustic startle response via electrical skin conductivity in humans to measure the startle responses (Takahashi et al. 2011).

PPI Statistical Analysis

A three-way repeated measures ANOVA was used to analyze PPI performance, with the between subject factors neonatal drug treatment (Poly IC/Saline) and diet (Experiment 2: PD2024/Control; Experiment 3: PD340 low dose, high dose, and control) and decibel level (73, 76, 82 dB) was the repeated measures factor. Days of testing were averaged together in adolescence (P44-46) and in adulthood (P60-66) as one mean for each subject. The Newman-Keuls was used as the post hoc test to analyze any statistically significant interactions ($p=0.05$).

Immunohistochemistry (IHC) & Confocal Microscopy

Immunohistochemistry (IHC) was conducted on brain tissue collected from animals tested in Experiments 2 and 3. On P67-69, these animals were anesthetized and intracardially perfused with 4% paraformaldehyde (PFA). Brains were removed and stored in 20% sucrose for 48 hours and then transferred to a clean vial and stored at -80 °C. Tissue was coronally sectioned at 50 μm thickness using a Leica cryostat. The prefrontal cortex (PFC) and hippocampus (HPC) were sectioned and stored as free-floating sections in 0.1 M phosphate buffered saline (PBS) (pH 7.3) until IHC began.

Free-floating sections were washed four times in 0.1 M PBS (pH 7.3; 10 minutes each wash). Sections were permeablized with 0.4% Triton X-100 in PBS containing 0.5% bovine serum albumin (BSA) for 20 minutes. Sections were then blocked for 2 hours in PBS containing 10% normal donkey serum (Jackson ImmunoResearch Laboratories, West Grove, PA), 1% BSA,

and 0.4% Triton X-100. Tissue sections were then incubated overnight at 4 °C in PBS, 1% BSA, and 0.4% Triton X-100 with the primary antibody, Iba1 (1:1,000, catalog #019-19741) (Wako Chemicals USA, Richmond, VA), which was used to label microglial cells. The next day, sections were washed four times with 0.1 M PBS (pH 7.3; 10 minutes each). Sections were then incubated for twenty minutes in PBS containing 0.4% Triton X-100 and 0.5% BSA. The secondary antibody (1:200 dilution) used to emit fluorescence (AlexaFlour488 conjugated Anti-Rabbit IgG – Jackson ImmunoResearch Laboratories, West Grove, PA) was then added and sections were incubated for 2 hours in PBS with 1% BSA and 0.4% Triton X-100. Sections were then washed four times in 0.1 M PBS (pH 7.3; 10 minutes each) and then transferred to charged slides. A drop of Citiflour mounting medium (Ted Pella, Inc., Redding, CA) was added to the center of the tissue and coverglass was applied. The coverglass was sealed with clear nail polish the following day and remained protected from light exposure until confocal microscopy analysis.

Slides were examined at a magnification of 40x using a Leica TCS SP8 inverted confocal microscope. Specifications (Smart Gain = 509.5 V, Smart Offset = -0.11%, Zoom = 2.0, Objective Lens = 20x/0.75 Dry, Resolution = 1024 x 1024) remained constant throughout all imaging. A 30.1 μm thickness was captured per image, and eight images were captured (4 PFC, 4 HPC images) per animal. Images were saved in TIFF format to be quantified using the National Institutes of Health (NIH) ImageJ software. The NIH ImageJ software was bundled with Java 1.8.0_172 for Mac OS X, https://imagej.nih.gov/ij/ to obtain statistical analysis and evaluate overall image fluorescence (Figure 4). The rectangular box tool from ImageJ was used to select the entire TIFF image. Integrated Density from ImageJ was used to obtain quantitative

values for overall image fluorescence determination. Each TIFF image generated one data point thereby giving eight representative data points combined from the two brain regions.

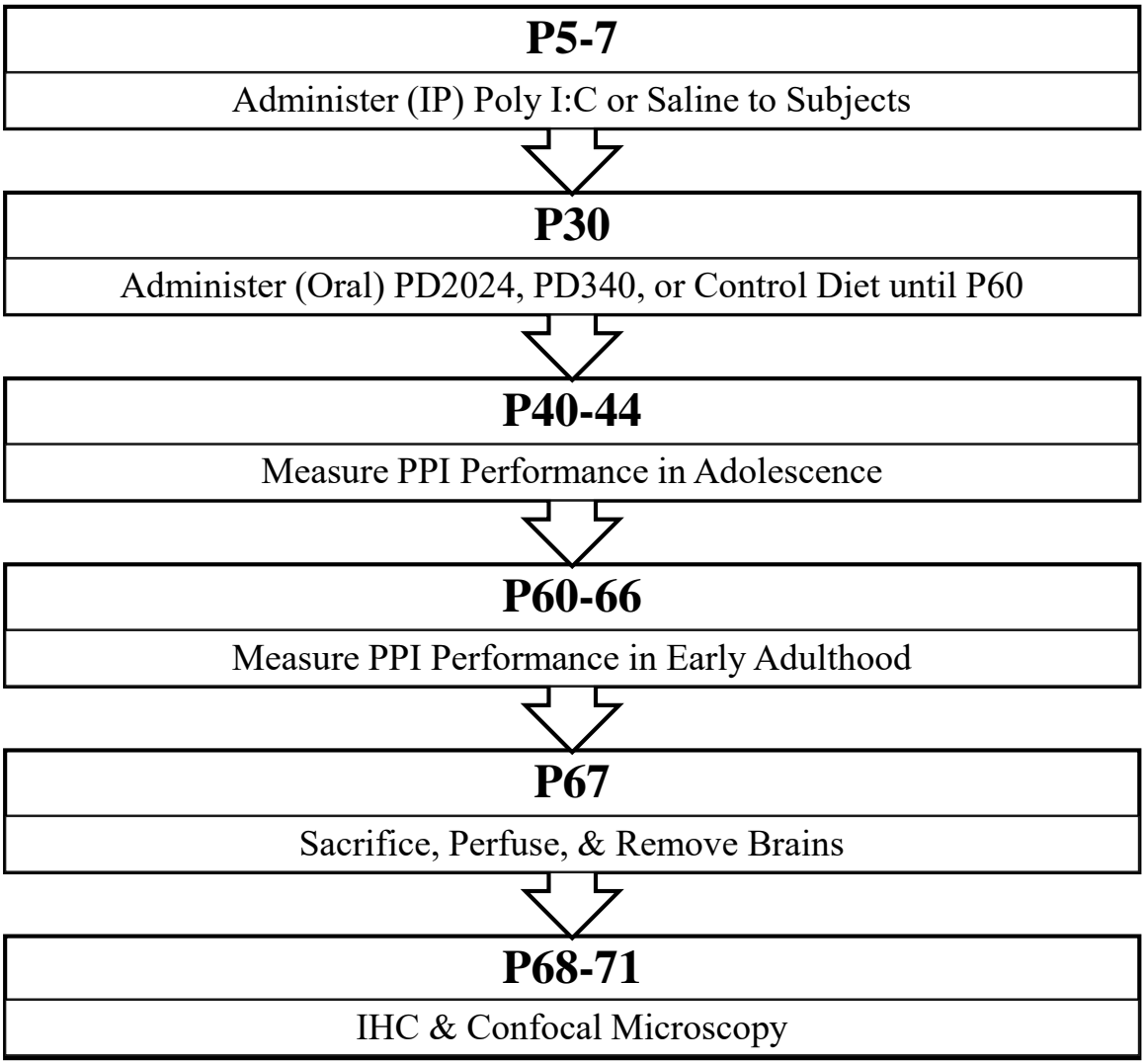

Figure 1: Experiments 2 & 3 Flowchart. *Flowchart outlining schedule and associated testing procedures for these experiments.*

Microglia Cell Count Analysis

Activated microglial cell counts were determined by two outside observers blind to the study. The two participants each counted the total amount of fluorescently activated microglial cells per image and the average number between the two observers was recorded. There was not

significant variance in cell counts between the two observers. Each TIFF image generated one data point, and eight TIFF images (4 PFC, 4 HPC) were taken, thus generating eight data points per animal. Cell counts were analyzed with a one-way analysis of variance (ANOVA). The one-way ANOVA was selected to analyze each brain area and determine significant differences between the variance of cell counts (dependent variable) by drug/treatment manipulation (independent variable). The Newman-Keuls post hoc test (p=0.05) was used to analyze significant interactions (p=0.05).

Microglial Cell Body Fluorescence Analysis

In addition to cell counts, we also sampled cell body fluorescence statistics from the Integrated Density tool. These quantifications were analyzed with a one-way analysis of variance (ANOVA). The one-way ANOVA was selected to analyze each brain area and determine significant differences between the variance of sampled cell body fluorescence (dependent variable) by drug/treatment manipulation (independent variable). The Newman-Keuls post hoc test (p=0.05) was used to analyze significant interactions (p=0.05).

Finally, we also sampled overall image fluorescence statistics from the Integrated Density tool, which was analyzed with a one-way analysis of variance (ANOVA). The one-way ANOVA was selected to analyze each brain region and determine significant differences between the variance of overall image fluorescence (dependent variable) by drug/treatment manipulation (independent variable). The Newman-Keuls post hoc test (p=0.05) was used to analyze significant interactions (p=0.05).

CHAPTER 3

RESULTS

Experiment 1: TNFα ELISA Protein Levels

TNF*α* protein levels (pg/mg) are presented as a function of drug treatment group in Figure 2. An independent t-test was used to analyze each brain area. For the HPC, the independent t-test revealed a significant main effect of neonatal drug treatment in the dorsal hippocampus $t(12) = 2.21$, $p<0.5$ as well as the ventral hippocampus $t(12) = 3.16$, $p<0.01$ with Poly I:C treatment producing increases in both regions. Though elevated in the frontal cortex, there was not a significant difference in TNFα levels due to high variability in the Poly I:C treated group (N=5). Therefore, it appears that TNF*α* protein levels were significantly increased by neonatal Poly I:C treatment in the hippocampal brain region.

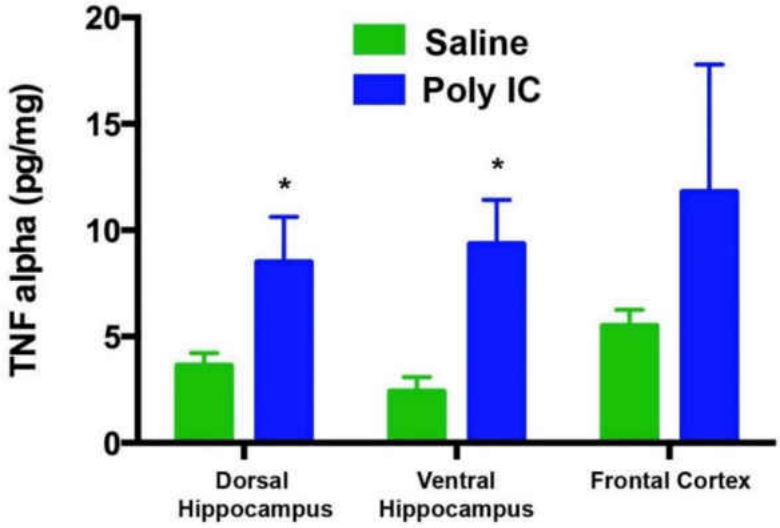

Figure 2: TNFα Protein Levels Following Saline or Poly I:C Administration between P5-7. *Mean TNFα protein levels (pg/mg) in both the dorsal and ventral hippocampus and frontal cortex following treatment with either saline (0.9% NaCl – N=8) or Poly I:C (2 mg/kg – N=9) from postnatal days 5-7. Data represents the results of Experiment 1 (mean ± SEM). Data were analyzed by an independent t-test ($p<0.05$).*

Experiment 2: PD2024 - Body Weights

Animal body weights following treatment with either neonatal saline or Poly I:C treatment and with either PD2024 or control diet present were assessed from P30-60 (Table 1). We analyzed the data in 10-day time intervals (P30, P40, P50, P60) to determine if significant differences between neonatal or dietary manipulation groups were present. A two-way ANOVA (neonatal drug treatment x diet) showed no significant main effects or interactions. Therefore, dietary manipulation and/or drug treatment had no significant effect nor did they interact on normal body weight or weight gain over the adolescent period.

Table 1: Animal Body Weights Across Experiment 2 Groups from P30-60. *Mean body weight (in grams) across neonatal drug treatments and diets from postnatal days 30-60 measured at 10-day time intervals (Saline/Control N=5, Poly IC/Control N=6, Saline/PD2024 N=5, Poly IC/PD2024 N=6) (mean ± SEM). Data were analyzed by a two-way ANOVA.*

Neonatal Drug Treatment	Diet	P30 Weight (g)	P40 Weight (g)	P50 Weight (g)	P60 Weight (g)
Saline	Control	87.7 ± 6.30	159.0 ± 7.67	223.4 ± 5.21	278.3 ± 7.2
Poly IC	Control	98.9 ± 2.29	170.3 ± 4.1	244.0 ± 4.25	304.1 ± 5.7
Saline	PD2024	100.3 ± 4.6	165.7 ± 5.6	226.0 ± 5.1	278.3 ± 7.1
Poly IC	PD2024	100.3 ± 2.65	168.4 ± 6.14	240.0 ± 9.8	294.4 ± 12.8

Experiment 2: PD2024 - Prepulse Inhibition Performance

Auditory sensorimotor gating measured by prepulse inhibition (PPI) performance is presented in Figure 3A for adolescent rats and 3B for adults. PPI is presented as a function of neonatal drug treatment and dietary condition. In adolescents (Figure 3A), a three-way ANOVA revealed a significant two-way interaction of neonatal drug treatment x diet $F(1,32) = 16.72$, $p<0.001$. At all prepulse intensities, Saline/Control and Poly IC/PD2024 groups were equivalent, and showed significantly improved PPI performance compared to the Poly IC/Control and

Saline/PD2024 groups. Furthermore, the Saline/Control group and Poly IC/PD2024 groups were statistically equivalent across all prepulse decibel levels.

In adulthood (Figure 3B), a three-way ANOVA showed a significant main effect of diet $F(1,32) = 6.24$, $p<0.01$ and a significant interaction of neonatal drug treatment x diet $F(1,32) = 24.81$, $p<0.001$. PPI deficits were found to be alleviated by PD2024 that was produced by neonatal Poly I:C treatment and Poly IC/PD2024 was equivalent to Saline/PD2024 and Saline/Controls. Poly I:C/Control animals demonstrated deficits at all three decibel levels.

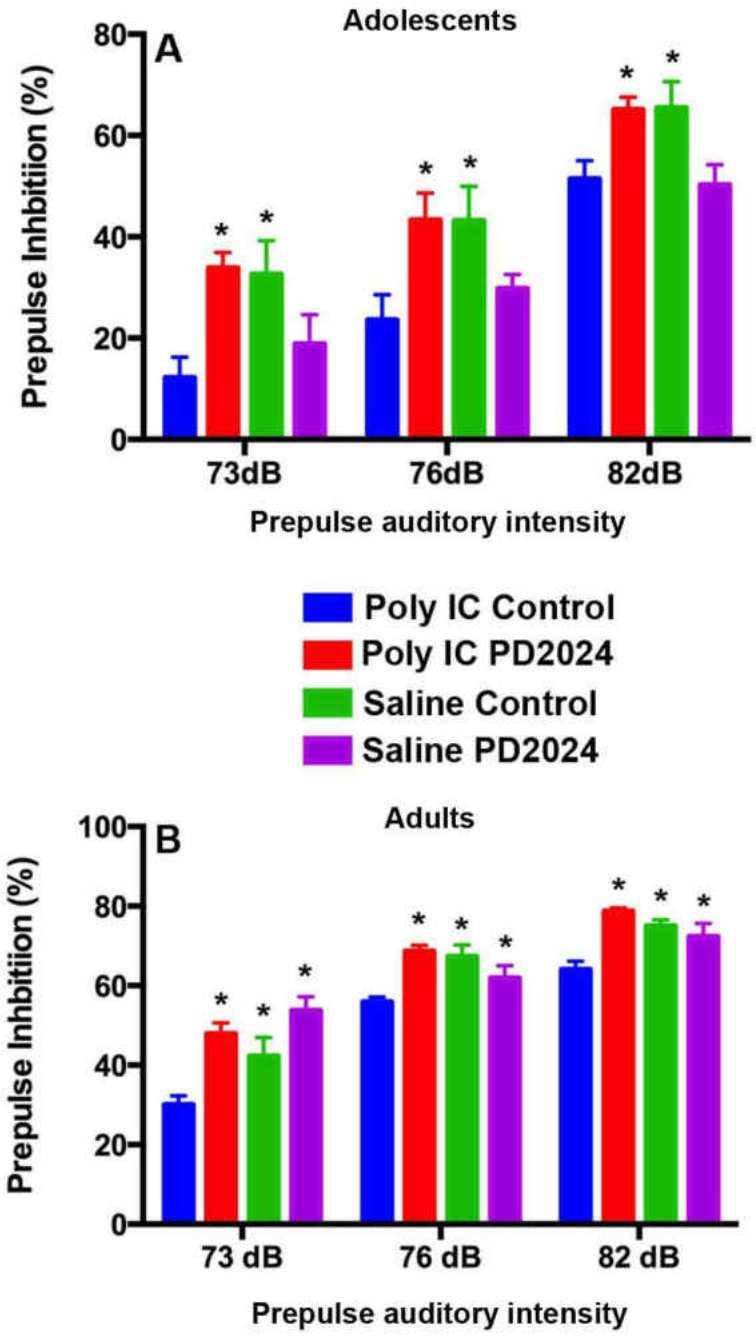

Figure 3: PPI Performance in Adolescents and Adults Across Experiment 2 Groups. *Mean PPI percentages across all prepulse trails (73 dB, 76 dB, and 82 dB) in both adolescence (P44-46) and early adulthood (P60-66) following treatment with Poly I:C or saline and with either PD2024 or control diet present (Saline/Control N=5, Poly IC/Control N=6, Saline/PD2024 N=5, Poly IC/PD2024 N=6) (mean ± SEM). Data were analyzed by a three-way ANOVA ($p<0.05$).*

Experiment 2: PD2024 - Immunohistochemistry

There were no significant effects in microglial activation cell counts and overall fluorescence. However, there were significant group differences on the microglial cell body analysis. Microglial cell body activation is presented in the prefrontal cortex (PFC), CA1 and CA3 regions of the hippocampus (HPC) as a function of neonatal drug treatment and dietary condition in Figure 4. A one-way ANOVA revealed a significant main effect of group in the PFC: $F(3,15) = 8.0$, $p<0.003$, HPC CA1: $F(3,16) = 8.1$, $p<0.003$, and HPC CA3: $F(3,16) = 12.8$, $p<0.001$. Animals neonatally administered Poly I:C demonstrated a significant increase in fluorescence intensity in microglial cells regardless of brain region. PD2024 reduced microglia activation to control levels, suggesting PD2024 is effective in decreasing microglial cell activation in Iba1 positive cells.

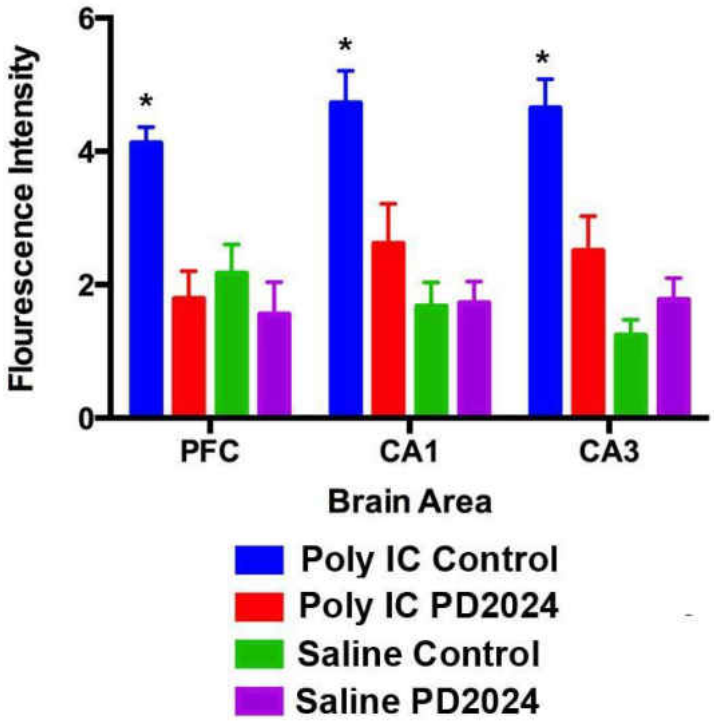

Figure 4: Microglial Cell Activation in the PFC and HPC Across Experiment 2 Groups. *Microglial cell activation as measured by fluorescence intensity in the prefrontal cortex (PFC) and CA1/CA3 regions of the hippocampus (HPC) following neonatal drug treatment (Saline or Poly I:C) and/or dietary manipulation (PD2024 or Control) (Saline/Control N=5, Poly IC/Control N=6, Saline/PD2024 N=5, Poly IC/PD2024 N=6) (mean ± SEM). Data were assessed by a separate one-way ANOVA used to analyze each brain area.*

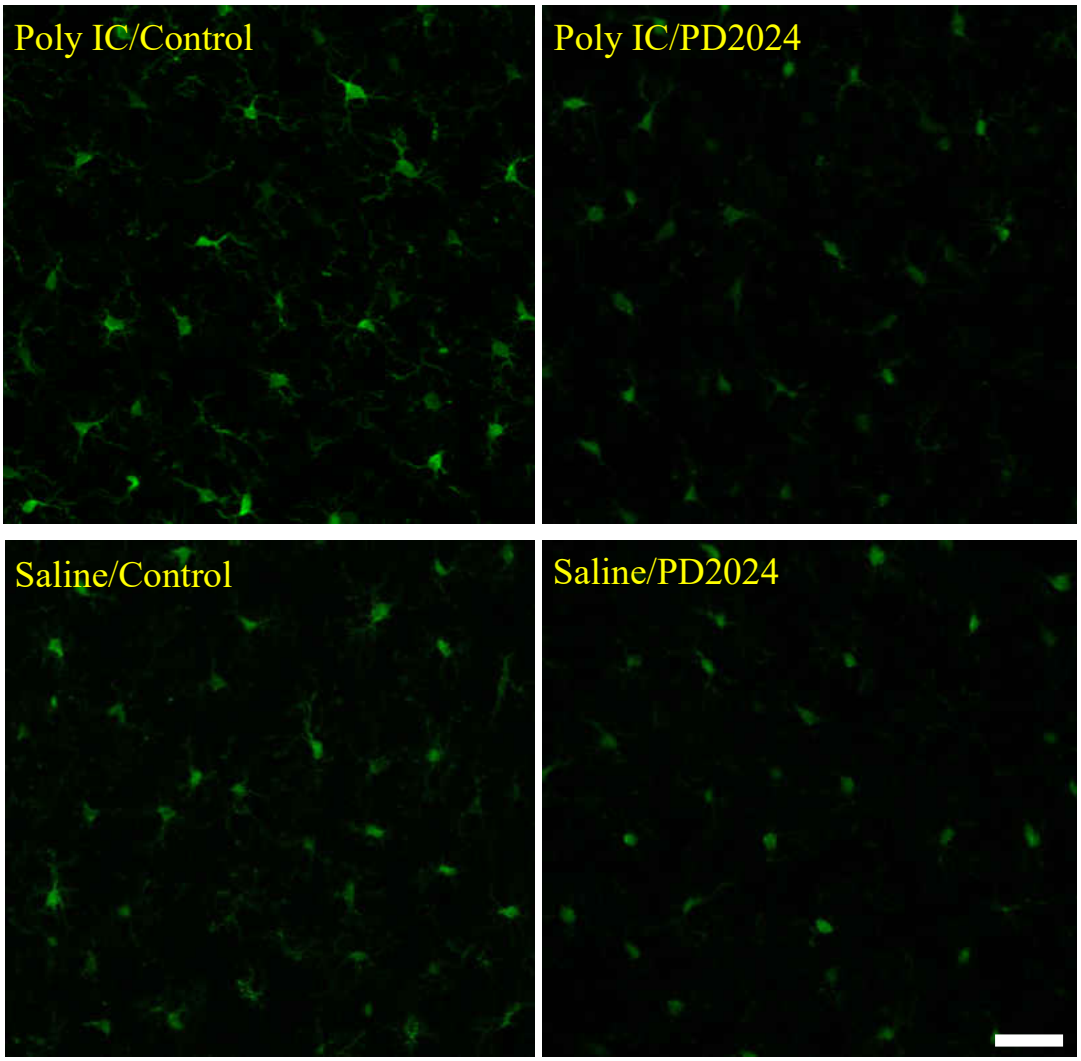

Figure 5: Representative Images of Flourescently Labeled Microglia Cells Across Experiment 2 Groups in the Prefrontal Cortex. *One representative image from each group (Poly IC/Control, Poly IC/PD2024, Saline/Control, Saline/PD2024) for GFP-labeled microglia cells in the PFC (Scale bars, 100 μm).*

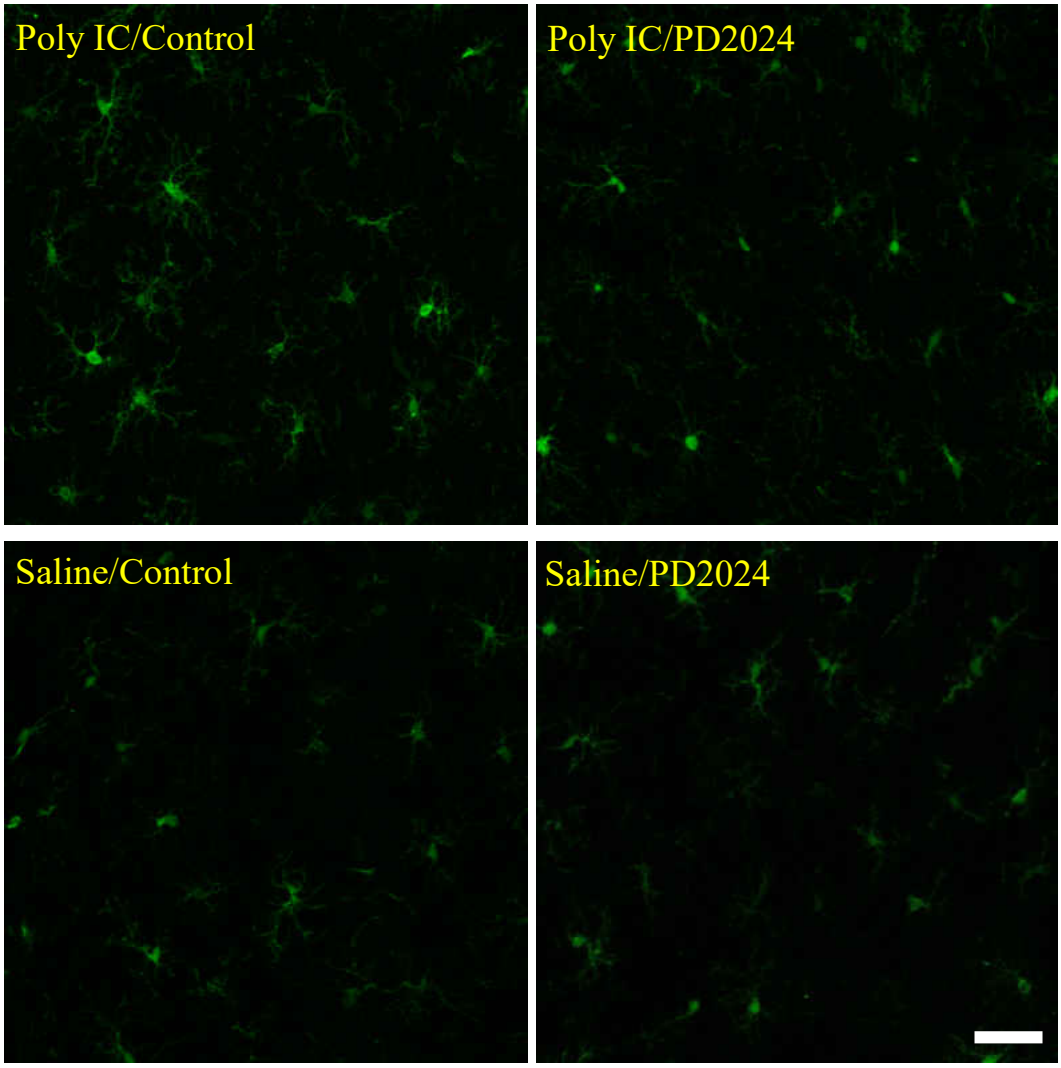

Figure 6: Representative Images of Flourescently Labeled Microglial Cells Across Experiment 2 Groups in the Hippocampus. *One representative image from each group (Poly IC/Control, Poly IC/2024, Saline/Control, Saline/PD2024) for GFP-microglia cells in the HPC (Scale bars, 100μm).*

Experiment 3: PD340 - Body Weights

Animal body weight and weight gain following neonatal treatment with either saline or Poly I:C and with either PD340 (10 mg/kg or 30 mg/kg) or control diet were assessed from P30-60 and presented in Table 2. A two-way ANOVA revealed a significant main effect of group $F(5,36) = 3.4$, $p<0.01$ and a significant group x day interaction $F(15,108) = 8.41$, $p<0.001$. A Newman-Keuls post hoc test revealed all five groups were equivalent from P30-50, but several

group differences emerged at P60. The Saline/Control and Poly IC/Control groups were equivalent and had significantly greater body weights than all other groups. These results thereby indicate PD340 decreases weight gain in neonatal saline and Poly I:C treated animals. Future work could investigate PD340's ability to be used as an adjunctive with an antipsychotic to ameliorate weight gain that accompanies currently associated pharmacological treatment.

Table 2: Animal Body Weights Across Experiment 3 Groups from P30-60. *Mean body weight (in grams) across neonatal drug treatments and diets from postnatal days 30-60 measured at 10-day time intervals (Saline/Control N=5, Poly IC/Control N=6, Saline/PD340 – 10 mg/kg N=5, Saline/PD340 – 30 mg/kg N=5, Poly IC/PD340 – 10 mg/kg N=5, Poly IC/PD340 – 30 mg/kg N=5) (mean ± SEM). Data were analyzed by a two-way ANOVA (* indicates p<0.05).*

Neonatal Drug Treatment	Diet	P30 Weight (g)	P40 Weight (g)	P50 Weight (g)	P60 Weight (g)
Saline	Control	96.3 ± 4.05	179.7 ± 5.25	223.4 ± 3.6	**320.3 ± 5.9***
Poly IC	Control	97.0 ± 1.97	175.5 ± 4.1	247.6 ± 4.5	**303.3 ± 5.1***
Saline	PD340 10 mg/kg	98.12 ± 3.9	175 ± 4.0	244.4 ± 4.5	294 ± 5.2
Saline	PD340 30 mg/kg	93.1 ± 3.0	159.1 ± 5.4	223.8 ± 5.5	267.6 ± 5.1
Poly IC	PD340 10 mg/kg	96.87 ± 3.9	172 ± 6.1	238.88 ± 5.4	287.75 ± 6.4
Poly IC	PD340 30 mg/kg	95.38 ± 4.4	161.1 ± 5.3	227.4 ± 7.4	273.5 ± 7.0

Experiment 3: PD340 - Prepulse Inhibition Performance

Auditory sensorimotor gating as measured by prepulse inhibition (PPI) performance in both adolescence and adulthood is presented in Figure 7. PPI is presented as a function of neonatal drug treatment and dietary condition. In adolescents, a three-way ANOVA revealed a significant main effect of diet $F(2,41) = 3.90$, $p<0.02$ and a significant two-way interaction of

neonatal drug treatment x diet $F(2,41) = 4.48$, $p<0.01$. A Newman-Keuls post hoc test revealed that the Poly IC/Control group was significantly lower than both PD340 treated groups as well as the Saline/Control group at 73 and 82 dB. The PD340 (30 mg/kg) group was equivalent to the Poly IC/Control group at 76 dB.

In adulthood, a three-way ANOVA revealed a significant main effect of diet $F(2,41) = 4.73$, $p<0.01$ and a significant interaction of neonatal drug treatment x diet $F(2,41) = 5.38$, $p<0.008$. A Newman-Keuls post hoc test revealed that the Poly IC/Control group was significantly lower than both PD340 treated groups and the Saline/Control group at all three prepulse (73, 76, and 82) decibel levels. Regardless of dose, these results demonstrate PD340 alleviated sensorimotor gating deficits generated by neonatal Poly I:C treatment in adults.

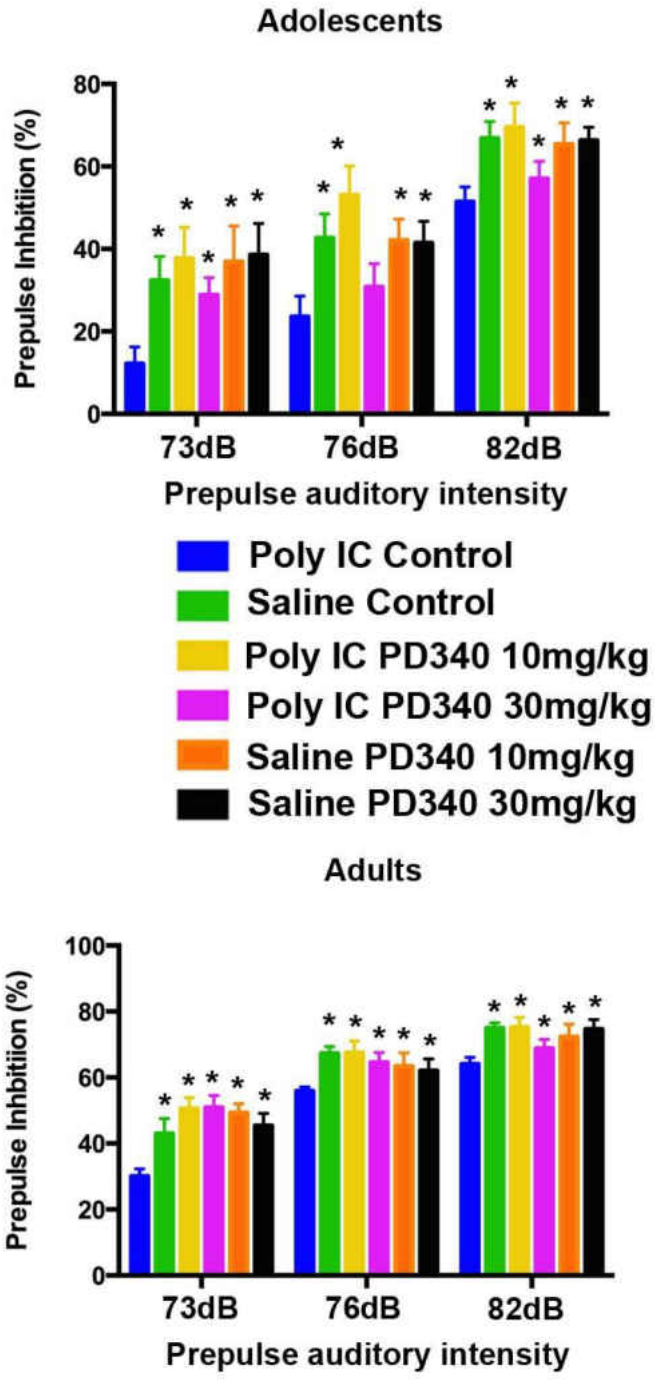

Figure 7: PPI Performance for Adolescents and Adults Across Experiment 3 Groups. *Mean PPI percentages across all prepulse trials (73 dB, 76 dB, and 82 dB) in both adolescence (P44-46) and early adulthood (P60-66) following treatment with Poly I:C or saline and with either PD340 (10 mg/kg or 30 mg/kg) or control diet present (Saline/Control N=5, Poly IC/Control N=6, Saline/PD340 – 10 mg/kg N=5, Saline/PD340 – 30 mg/kg N=5, Poly IC/PD340 – 10 mg/kg N=5, Poly IC/PD340 – 30 mg/kg N=5) (mean ± SEM). Data were analyzed by a three-way ANOVA ($p<0.05$).*

Experiment 3: PD340 - Immunohistochemistry

There were significant group differences in microglial cell activation in all analyses, including cell count, sampled cell body fluorescence, and overall image fluorescence. Cell counts are presented as a function of the number of Iba1 positive cells as a function of brain area and group in Figure 8. A two-way ANOVA revealed a significant main effect of diet in the PFC $F(2,42) = 6.63$, $p<0.01$, but there were no significant main effects or interactions in the CA1 or CA3 regions of the hippocampus. In the PFC, there was an overall increase in Iba1 positive cells produced by PD340 in comparison to control diet. PD340 increased the number of Iba1 positive cells regardless of dose.

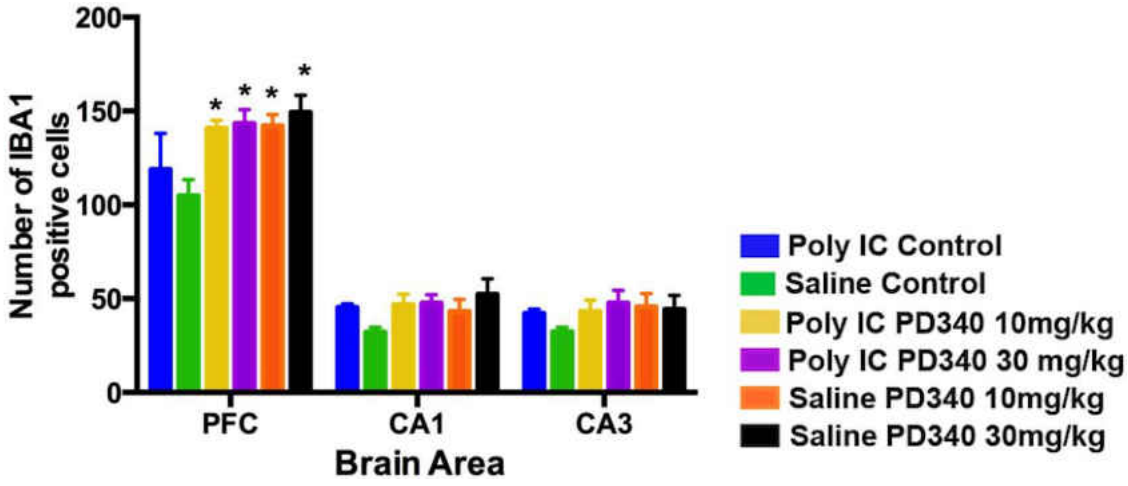

Figure 8: Iba1 Positive Cell Counts in the PFC and HPC Across Experiment 3 Groups. *Microglial cell counts in the prefrontal cortex (PFC) and CA1/CA3 regions of the hippocampus (HPC) following neonatal drug treatment (Saline or Poly I:C) and/or dietary manipulation (PD340 – 10 mg/kg or 30 mg/kg) (Saline/Control N=5, Poly IC/Control N=6, Saline/PD340 – 10 mg/kg N=5, Saline/PD340 – 30 mg/kg N=5, Poly IC/PD340 – 10 mg/kg N=5, Poly IC/PD340 – 30 mg/kg N=5) (mean ± SEM). Data were assessed by a two-way ANOVA used to analyze the number of Iba1 positive cells as a function of brain area and group.*

Cell body fluorescence is presented as a function of brain area and groups in Figure 9. A two-way ANOVA for cell body fluorescence revealed significant main effects of diet in both

CA1 F(1,41) = 5.47, p<0.02 and CA3 F(1,41) = 6.77, p<0.01, and significant interactions of neonatal drug treatment x diet in both CA1 F(2,41) = 10.03, p<0.001 as well as CA3 F(2,41) = 15.44, p<0.001. In both hippocampal areas, CA1 and CA3, Poly IC/Controls demonstrated higher amounts of cell body fluorescence intensity than all other groups. In the PFC, there was a significant main effect of diet F(2,41) = 8.97, p<0.001. Surprisingly, PD340 produced an increase in fluorescence intensity regardless of neonatal drug treatment as compared to saline controls, but similar to cell counts in the PFC, these groups were not different from Poly IC/Controls, and Poly IC/Controls were significantly greater than Saline/Controls.

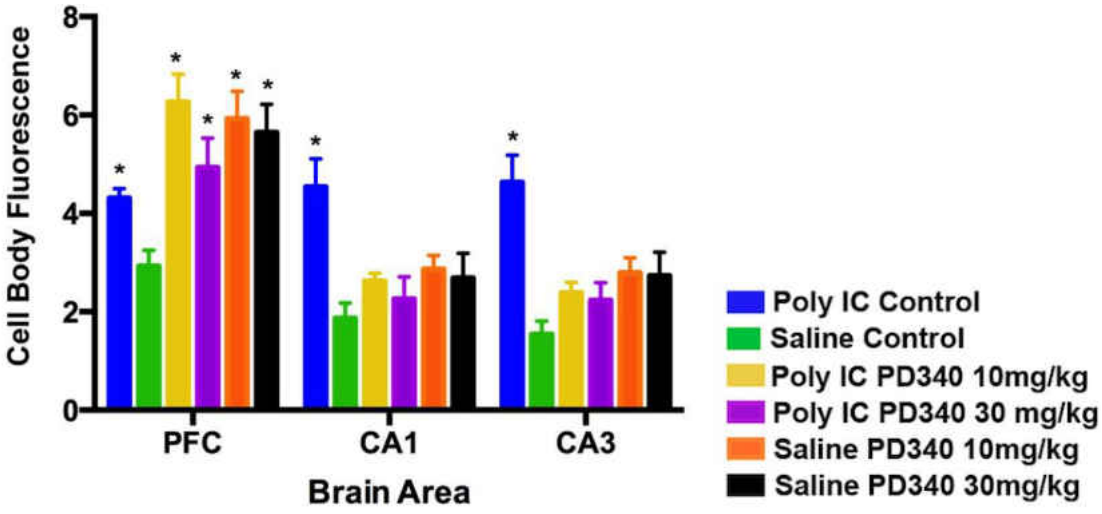

Figure 9: Microglial Cell Activation in the PFC and HPC Across Experiment 3 Groups. *Microglial cell activation as measured by sampled cell body fluorescence intensity in the prefrontal cortex (PFC) and CA1/CA3 regions of the hippocampus (HPC) following neonatal drug treatment (Saline or Poly I:C) and/or dietary manipulation (PD340 – 10 mg/kg or 30 mg/kg) (Saline/Control N=5, Poly IC/Control N=6, Saline/PD340 – 10 mg/kg N=5, Saline/PD340 – 30 mg/kg N=5, Poly IC/PD340 – 10 mg/kg N=5, Poly IC/PD340 – 30 mg/kg N=5) (mean ± SEM). Data were examined by a two-way ANOVA used to analyze the effects of neonatal drug treatment and diet.*

Overall image fluorescence is presented as a function of brain area and groups in Figure 10. A two-way ANOVA for overall image fluorescence revealed a significant main effect of diet

for the PFC $F(2,41) = 18.0$, $p<0.001$, but no other significant main effects or interactions in the CA1 or CA3 brain regions. Supporting our findings in the PFC in cell body fluorescence, PD340 produces an increase in microglial activation in this region regardless of neonatal drug treatment as compared to controls, however in this case, there were no significant differences between Poly IC/Controls and Saline/Controls in any brain region.

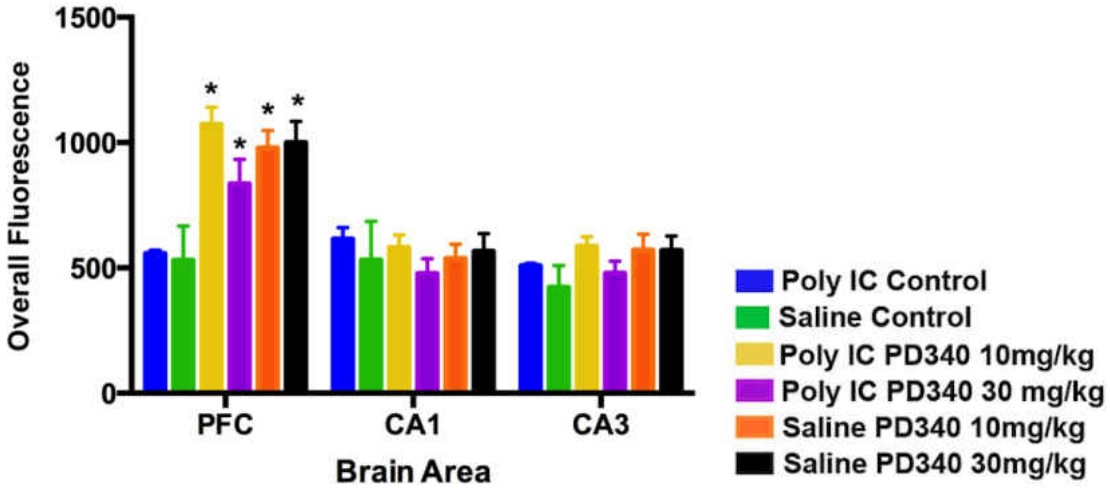

Figure 10: Overall Image Fluorescence Across Experiment 3 Groups in the PFC and HPC. *Overall image activation as measured by fluorescence intensity in the prefrontal cortex (PFC) and CA1/CA3 regions of the hippocampus (HPC) following neonatal drug treatment (Saline or Poly I:C) and/or dietary manipulation (PD340 – 10 mg/kg or 30 mg/kg) (Saline/Control N=5, Poly IC/Control N=6, Saline/PD340 – 10 mg/kg N=5, Saline/PD340 – 30 mg/kg N=5, Poly IC/PD340 – 10 mg/kg N=5, Poly IC/PD340 – 30 mg/kg N=5) (mean ± SEM). Data were assessed using a two-way ANOVA, which was used to analyze the effects of group and brain area.*

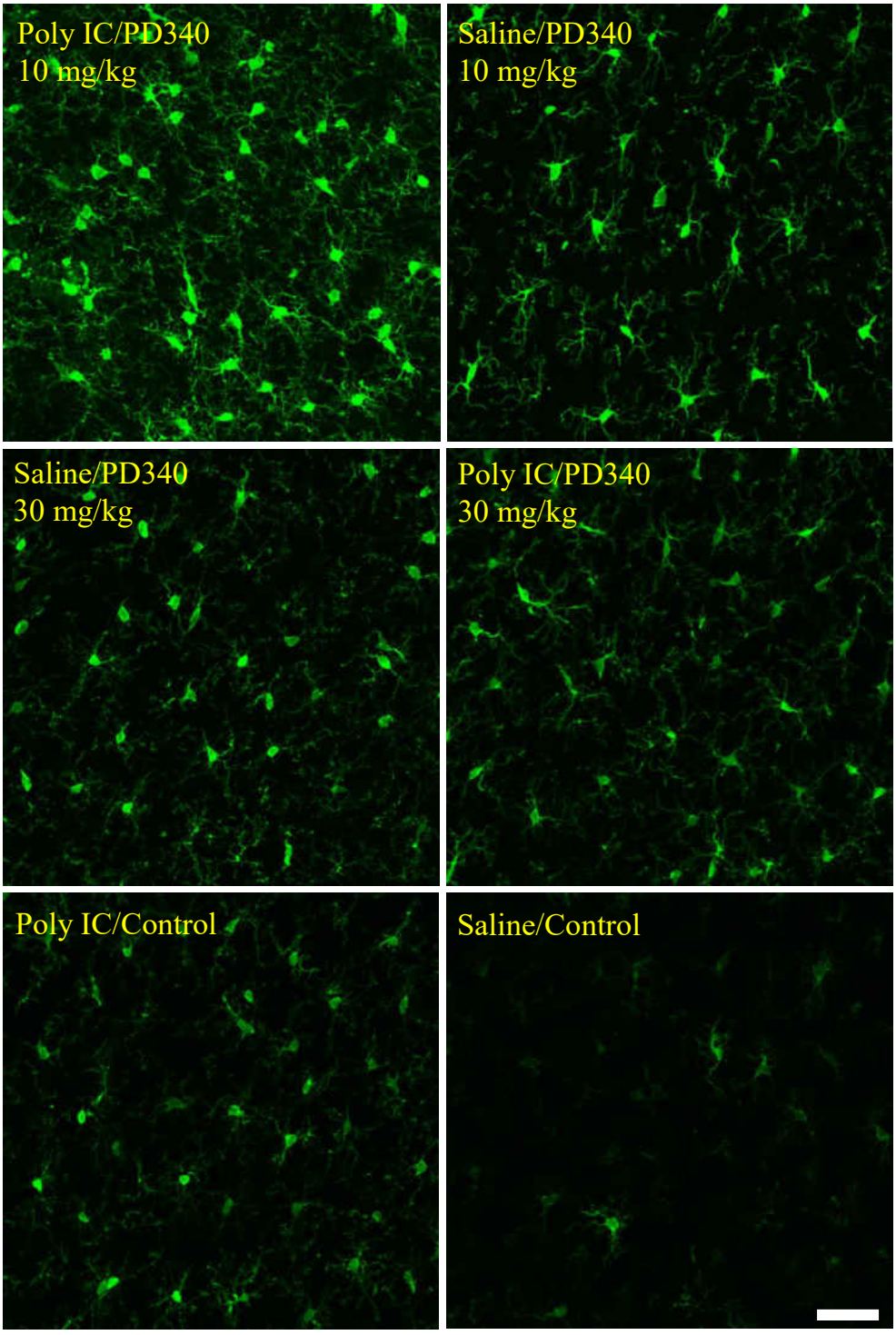

Figure 11: Representative Images of Iba1 Labeled Microglia Cells Across Experiment 3 Groups in the Prefrontal Cortex. *One image from each group (Poly IC/Control, Saline/Control, Poly IC/PD340 – 10 mg/kg, Poly IC/PD340 – 30 mg/kg, Saline/PD340 – 10 mg/kg, Saline/PD340 – 30 mg/kg) used to represent cell body fluorescence intensity in the PFC (Scale bars, 100 μm).*

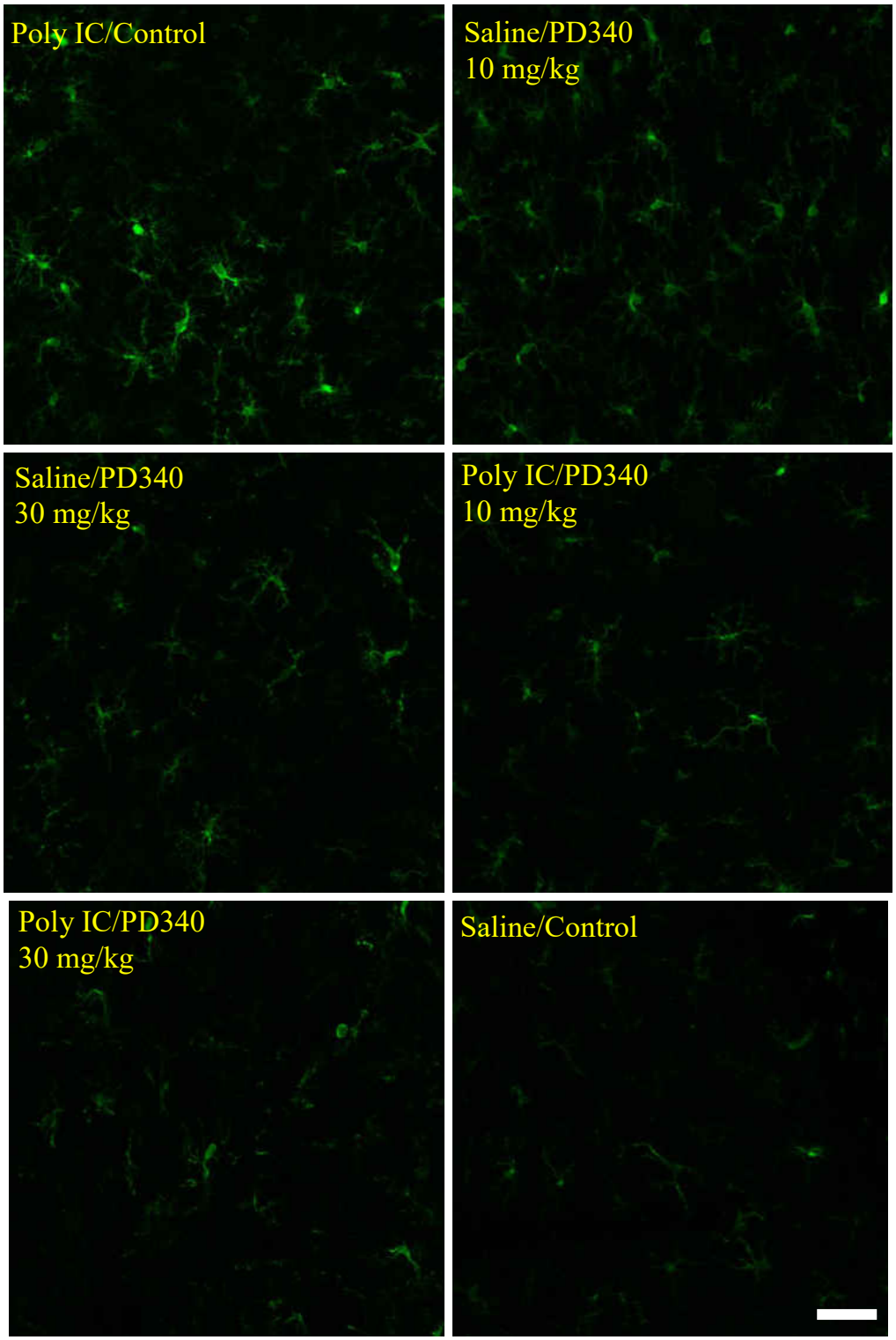

Figure 12: Representative Images of Iba1 Labeled Microglia Cells Across Experiment 3 Groups in the Hippocampus. *One image from each group (Poly IC/Control, Saline/Control, Poly IC/PD340 – 10 mg/kg, Poly IC/PD340 – 30 mg/kg, Saline/PD340 – 10 mg/kg, Saline/PD340 – 30 mg/kg) used to represent cell body fluorescence intensity in the HPC (Scale bars, 100 μm).*

CHAPTER 4

DISCUSSION

In this study, we were able to show neonatally administered Poly I:C increases neuroinflammation within the brain and generates behavioral abnormalities in adolescence and early adulthood, thereby validating the use of the Poly I:C model to mimic SCZ. Poly I:C was also shown to generate reactive microglia in the PFC and HPC, two areas shown to be affected in those diagnosed. When a novel TNFα modulator was administered through the diet from early adolescence to adulthood, sensorimotor gating deficits were alleviated and microglial cell activation decreased, although not across all brain regions examined. Our two novel TNFα modulators did not increase weight gain, with one of the modulators, PD340, actually significantly reducing weight gain when compared to the neonatal Poly I:C animals. This is an important effect, because weight gain is a common side effects of antipsychotics. Both modulators used in this study were proven to be safe and well-tolerated in all subjects, thereby further demonstrating excellent preclinical data for the continued development of novel anti-inflammatory drugs to be investigated as treatment for SCZ.

Poly I:C Administration Leads to Increased TNF-Alpha Protein Levels in the Brain

In Experiment 1, the analysis of TNFα protein revealed that neonatal Poly I:C administration significantly increased levels of the pro-inflammatory cytokine, TNFα, compared to controls in the two subregions of the hippocampus proper, CA1 and CA3. The hippocampus is a brain area that is critically important in prepulse inhibition, as past work has demonstrated deficits are present in those diagnosed with SCZ (Braff et al. 1978; Caine et al. 1992; Swerdlow

et al. 2008) and the hippocampus is also critical in PPI in rats (Zhang et al. 2002). In addition, the hippocampus plays a critical role in cognition as well as in behaviors related to the negative symptoms often observed in schizophrenia (Howes et al. 2015).

There was a large variation in TNFα protein levels across animals in the PFC, and a significant group difference was not observed. Overall, these results confirm neonatal Poly I:C is successful in initiating activation of TNFα in the brain. Increases in TNFα lead to activation of the immune system (Cope 2002), which ultimately contributes to elevated inflammation within certain brain regions later in life (Clark et al. 2005). The increase in TNFα at least in part validates the use of the Poly I:C rodent model of SCZ for generating neural inflammatory abnormalities not observed in healthy/control animals.

Neonatal Poly I:C Treatment Results in Behavioral Deficits in Adolescence and Adulthood

The analyses of auditory sensorimotor gating as measured via prepulse inhibition (PPI) in Experiments 2 and 3 revealed that neonatal Poly I:C administration resulted in deficits in adolescence and early adulthood. These findings are consistent with past studies (Ozawa et al. 2006; Osborne et al. 2017) that showed cognitive and sensorimotor gating deficits result from early developmental Poly I:C treatment, consistent with SCZ symptoms. Though not absolutely equivalent to control PPI intensities, PD2024 treatment in animals administered Poly I:C were similar to controls across all prepulse levels. This is consistent with previous work by Oh et al. (2017) that showed an anti-inflammatory compound such as Swertisin (plant-derived C-glucosylflavone) attenuated PPI deficits in mice generated by dizocilpine (MK-801), a non-competitive NMDA receptor antagonist.

In addition, these behavioral findings along with an increase in TNFα protein suggest that behavioral deficits that follow early Poly I:C treatment appear to correlate with microglial activation in the hippocampus. These results are consistent with previous work that showed neonatal or maternal Poly I:C administration results in structural abnormalities via reduced hippocampal and prefrontal cortex cortical volumes in directly treated or maternally administered Poly I:C offspring (Ribeiro 2014; Buschert et al. 2016; Meehan et al. 2017). Mechanisms to compensate for the reduction in cortical volumes includes an elevated neuroinflammatory response, as we show later. We did not verify changes in hippocampal neuronal morphology in this study, but past work (Piontkewitz et al. 2012) has shown that maternal Poly I:C treatment resulted in deficits in neurogenesis, disturbed micro-vascularization, and elimination of parvalbumin-expressing interneurons in the hippocampus.

Neonatal Poly I:C Generates Reactive Microglia in the PFC and HPC

Analyses from Experiments 2 and 3 generally revealed neonatal administration of the immunostimulant, Poly I:C, increases microglial cell activation in both the prefrontal cortex and hippocampus as quantified by sampled cell body fluorescence. In Experiment 2, it was found that Poly I:C substantially activates microglia as compared to control animals regardless of brain region. Experiment 3 further confirmed this when higher intensities of cell body fluorescence were found in the HPC, although the measure of overall image florescence did not reveal any significant difference between Poly IC/Controls and the saline/control group. These results combined indicate that Poly I:C not only increases neural TNFα protein levels, but also regulates the activation of microglial cells. These findings are consistent with the behavioral deficits in PPI

performance observed in animals neonatally treated with the synthetic virus, again validating the use of neonatal Poly I:C inoculation to mimic SCZ behavioral and neurological symptomatology.

Oral PD2024 Administered Through the Diet Does Not Affect Weight Gain

Administration of the TNFα modulator, PD2024, given orally through the diet did not have an effect on body weight or weight gain over the adolescent period. This is of particular importance regarding the treatment of SCZ with antipsychotic medications. One of the most prominent side effects of all current antipsychotic medications is weight gain. A recent review (Dayabandara et al. 2017) discussed the problem of dose-dependent antipsychotic-induced weight gain, which often leads to poor compliance and eventual discontinuation. Our data suggests a small TNFα modulator like PD2024 may be used adjunctively with an antipsychotic and thereby reduce the effective dose without producing a change in weight gain. However, in past work, Gabbita and colleagues have reported positive effects of PD2024 on several metabolic parameters in animals fed a high fat diet, including cumulative food intake, insulin, body fat, triglycerides and cholesterol (unpublished data). Although there were no effects in this particular study with PD2024, there have been positive data on PD2024 relative to weight gain in rats fed a high fat diet.

PD2024 Alleviates Auditory Sensorimotor Gating Deficits in Neonatal Poly I:C Animals

Auditory sensorimotor gating deficits measured using PPI were shown to be alleviated through the use of orally available PD2024. The results from Experiment 2 showed PPI performance improved in animals neonatally treated with Poly I:C that were later administered PD2024. In adolescents and across all prepulse intensities, the control and Poly IC/PD2024

groups were equivalent and significantly different than the Poly IC/Control and Saline/PD2024 groups. This suggests PD2024 alone in as early as adolescence can improve some of the associated cognitive symptoms that are hallmarks of SCZ. However, PD2024 generated deficits in the controls during adolescence, but the deficits were diminished in adulthood. The mechanisms underlying this result is not known, but suggests that PD2024 interacted at some level with adolescent brain development. Future investigation into the mechanism and how PD2024 created deficits in otherwise normal animals is still needed.

PD2024 Reduces Microglial Cell Activation Similar to Controls

Data obtained regarding microglial cell body activation revealed that PD2024 is effective in reducing microglial cell activation in the HPC. Experiment 1 showed neonatal Poly I:C elevates TNF-alpha protein levels particularly within the HPC, consistent and directly linked to increased microglia activation. We found a significant increase in microglia activation regardless of brain region that was reduced by PD2024 and was similar to control (Saline/Control) animals. This suggests PD2024 successfully modulates and down-regulates neural TNFα protein secretion, thus presumably decreasing the neuroinflammatory response produced by neonatal Poly I:C treatment. We observed in both the PFC and HPC that microglial cell activation was diminished by PD2024 and the remaining inflammation was comparable to what was found in control animals. These results further suggest the ability of PD2024 to modulate TNFα protein secretion in such a way that hinders the ability to activate microglia cells to their M1 state and become pro-inflammatory and neurotoxic.

Weight Gain is Decreased Following Dietary Administration of PD340

Dietary introduction of a bioavailable TNFα modulator (PD340) decreases weight gain in early adulthood. Our data demonstrated that PD340 did not affect body weight or normal weight gain during late adolescence, but does reduce weight gain in early adulthood, especially in the group given the higher dose of PD340 (30 mg/kg). Metabolism and distribution analyses are still needed to explain the mechanism behind how this occurs. However, this finding is significant and sheds valuable light for current investigations looking to reduce therapeutic doses of antipsychotic medications in order to reduce discontinuation and not result in weight gain as is often reported (Dayabandara et al. 2017). These results suggest PD340 could be used adjunctively with an antipsychotic to reduce the effective dosage and decrease weight gain that is associated with antipsychotic treatment.

PD340 Alleviates PPI Deficits in Neonatal Poly I:C Treated Rats

PPI deficits were alleviated using PD340, which was given orally through the diet. Neonatal treatment with Poly I:C was shown to create severe deficits in adolescence that were significantly different from controls at two of three prepulse decibel levels. At all decibel levels (except 76 dB), PD340 alleviated sensorimotor gating deficits that resulted from neonatal Poly I:C and performed similar to control animals. Our data suggests low dose (10 mg/kg) PD340 is more effective for improving PPI performance at all decibel levels. This is consistent with previous work by Long et al. (2006) that showed cannabidiol, a nonpsychoactive compound with anti-inflammatory properties, ameliorated PPI deficits produced by MK-801 administration. We also found that high dose (30 mg/kg) PD340 produced deficits at 76 dB in control

(Saline/PD340) animals during adolescence, but further investigation is needed to determine if neurotoxic effects occurred as a result of increased dosage.

In adults, neonatal Poly I:C again created PPI deficits at all auditory intensities. Our results again suggest low dose PD340 is more effective for alleviating deficits also observed in adulthood. The Poly IC/PD340 (10 mg/kg) group and Saline/Control groups were found to be approximately equivalent at all decibel levels. As such, PD340 treated animals performed as well as control animals, whereas animals neonatally administered Poly I:C had severe auditory sensorimotor gating deficits at two distinct time periods, hallmark to SCZ development and progression.

PD340 Increases Iba1 Immunoreactivity in the PFC

The results obtained from the Iba1 positive cell count analysis in Experiment 3 showed PD340 increased the amount of reactive microglia cells in the prefrontal cortex, but does not have an effect in the hippocampus. PD340 at both doses produced an increase in fluorescence intensity regardless of neonatal intervention in the PFC. Microglial activation, additionally measured in overall image fluorescence showed the same trend. PD340 increased fluorescence intensity in the PFC regardless of drug treatment as compared to controls. However, in the hippocampus, PD340 reduced fluorescence intensity as measured by sampled cell body fluorescence and overall image fluorescence that were similar to control animals. This is consistent with the results obtained in Experiment 2 with PD2024, suggesting the two modulators work in a similar fashion in the HPC, but may be having differential effects in the PFC. This is an interesting result, but we do not have any explanation as to why this may occur.

Further investigation is needed to determine how PD340 increases microglial cell activation in the prefrontal cortex at both low and high doses and with either saline or Poly I:C on board. It can be hypothesized that there may be a compensatory reaction taking place where the microglial cells in the PFC remain in a highly activated state and PD340 does not affect this activation. Future work could use different labeling markers designed to specifically target M1 and M2 microglia to elucidate these findings. The exact mechanism for how PD340 modulates TNFα protein could also be investigated (upon patent approval) to determine if modifications occur upstream in the pathway that eventually leads to changes in microglia.

Hippocampus Involvement in Auditory Sensorimotor Gating

Both novel TNFα modulators used in this study successfully alleviated deficits in PPI that were generated from neonatal administration of Poly I:C. PD2024 reduced microglial cell activation in the PFC and HPC, whereas PD340 only reduced microglia activation in the HPC. In fact, PD340 significantly increased Iba1 positive cells as well as microglia activation in the PFC. These results in combination strongly suggest that the hippocampus plays a larger role in mediating behavioral response measured in PPI than the PFC. This could be due to the HPC's role in memory formation and consolidation, thus suggesting a strong correlation between the HPC and sensorimotor gating abilities. Future investigation using fluorescent labeling markers to differentiate M1/M2 microglia would elucidate this claim. Characterization of microglia morphology in the HPC could also clarify the results that we found and further strengthen this claim.

CHAPTER 5

CONCLUSION AND FUTURE DIRECTIONS

Evidence continues to point towards an early infection as a factor that can dysregulate the development of the nervous system, thus increasing the possibility of developing a neurobehavioral disorder (Meyer 2014; Flinkkilä et al. 2016). This study investigated the use of the neonatal Poly I:C treatment, a rodent model of SCZ and the effectiveness of two novel TNF-alpha modulators for improving the abnormalities generated by the model. The model proved advantageous because behavioral abnormalities emerged in adolescence and adulthood, consistent with clinical observation and diagnosis of SCZ.

The effects of the modulators were assessed through the use of prepulse inhibition (PPI) to determine behavioral alterations and immunohistochemistical analysis of microglia in two brain areas important in PPI to determine neuroinflammatory levels. Results revealed that neonatal administration of Poly I:C activates the immune system to trigger elevated TNF-alpha protein levels and microglial activation that persists into adulthood. Behaviorally, neonatal Poly I:C resulted in PPI deficits, consistent with past work (Ozawa et al. 2006; Ribeiro 2014; Osborne et al. 2017). Treatment with both novel TNF-alpha modulators were shown to alleviate sensorimotor gating deficits in adolescence and adulthood, and decrease microglial cell activation within distinct regions of the brain known to mediate sensorimotor gating.

Future work will continue to assess novel pro-inflammatory cytokine inhibitors and modulators, such as those affecting TNF-alpha, and their effects on treating the many aspects of SCZ and other neuro-related diseases/disorders. Future studies will also investigate the use of PD2024 and PD340 used adjunctively in the Poly I:C and other rodent models of SCZ with

antipsychotic drugs. This work will further shed light into the development, pathophysiology, genetics, and environmental factors that contribute to developing schizophrenia. In doing so, we will continue to learn about currently unknown aspects of SCZ and look to determine effective treatment options for those diagnosed.

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Shelton H W, Gabbita S P, Gill W D, Burgess K C, Brown R W (2019). "The Effects of a Novel Inhibitor of Tumor Necrosis Factor Alpha on Prepulse Inhibition and Microglial Activation in Two Distinct Rodent Models Of Schizophrenia." Journal of Neuroinflammation (*ahead of press*)

Honors and Awards:

GPSA Transportation Funding Award Recipient ($1,000), 2018

Student-Faculty Collaborative Grant Recipient ($1,200), 2017