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The effects of a single acute and repeated intracerebroventricular infusions of propionic acid on locomotor activity and neuroinflammation in rats

Stacey Holbrook

The University of Western Ontario

Supervisor

Dr. Klaus-Peter Ossenkopp

The University of Western Ontario

Joint Supervisor

Dr. Martin Kavaliers

The University of Western Ontario

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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THE EFFECTS OF A SINGLE ACUTE AND REPEATED
INTRACEREBROVENTRICULAR INFUSIONS OF PROPIONIC ACID ON
LOCOMOTOR ACTIVITY AND NEUROINFLAMMATION IN RATS

(Thesis format: Integrated Article)

by

Stacey Holbrook

Graduate Program in Neuroscience

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

Dietary and gastrointestinal factors may contribute to the etiology of autism spectrum disorders (ASD). Propionic acid (PPA) is a short chain fatty acid that is an intermediary of fatty acid metabolism and a fermentation by-product of enteric bacteria. Using a single infusion (Chapter 2) and repeated infusions (Chapter 3), the temporal relationship between PPA-induced locomotor activity and astrocyte/microglial changes was demonstrated. Adult Long-Evans rats were centrally infused with 4 μ l of 0.26M PPA or 0.1M PBS vehicle once or once a week for four weeks. Locomotor activity was evaluated for 20 minutes following infusion and again several days later to assess drug-free activity. Rats were perfused at various time-points post-final infusion, and brain tissue was immunohistochemically (GFAP, CD68) analyzed. PPA produced rapid increases in locomotor activity and later, pronounced changes in glial cell activation. PPA's effects on neurotransmitters, calcium signaling, mitochondrial metabolism, and immune functioning are likely underlying mechanisms.

Keywords: Propionic Acid, Reactive Astrogliosis, Microglial Activation, Autism Spectrum Disorders, Thigmotaxis, Locomotor Activity

Co-Authorship Statement

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Chapter 1

General Introduction

1.1 Autism Spectrum Disorders

Autism Spectrum Disorders (ASD) are a family of neurodevelopmental disorders classified according to three main symptoms domains. Children display a combination of restricted, repetitive and stereotypic patterns of behaviour, coupled with hyperactivity and/or marked impairments in social functioning and communication. These characteristics can manifest in a variety of ways including avoidance of eye contact, failure to participate in make believe play, adherence to odd routines and rituals, hyperactivity, posturing and idiosyncratic language (*DSM-IV-R*; American Psychiatric Association, 2000).

With ASD now being diagnosed at a rate of 1 in 88 children in the United States (Centres for Disease Control and Prevention, 2012), research in the field has grown exponentially and a number of theories have been proposed. Historically, genetic origins have been the main investigative focus because of the increased risk that those with known genetic anomalies such as Fragile X syndrome, Down Syndrome and Tuberous Sclerosis (Trottier, Srivastava, & Walker, 1999) have of being diagnosed with ASD. More recently though, studies have shown discrepancies in concordance rates between monozygotic twins, and it appears that gene defects account for only 5-16% of ASD cases (Hu, Frank, Heine, Lee, & Quackenbush, 2006). Genetic models also tend to cause a more incomplete phenotype than that characteristic of ASD (Patterson, 2011). A simple genetic focus is, thus, unsatisfactory in explaining the striking increase in diagnoses. (Herbert, 2010). It is now widely accepted that environmental factors contribute substantially to the etiology, and numerous scientists have shifted their focus to various environmentally related factors (reviewed in Berg, 2009; Depino, 2013; Patterson, 2011).

1.2 Neurological Abnormalities

Findings of brain dysfunction in autistic individuals have been extensively reviewed. A host of neurological abnormalities have been noted using imaging techniques including hypoplasia in the vermis of the cerebellum (Courchesne et al., 2001) and hyperplasia of the grey and white matter in the frontal and temporal lobes (Carper, Moses, Tigue, & Courchesne, 2002). These authors also provided evidence for significant age effects in cerebral development with 2 and 3 year old ASD children showing initial hyperplasia

followed by a sharp decline in normal age-related development of the temporal grey and white matter. Specifically, in typically-developing controls, there was a 22% increase in temporal white matter volume, compared to a slight 2% increase in ASD patients. Similar contrasts were found in temporal grey matter volume (17% vs 1%; Carper et al., 2002). These findings emphasize the importance of focusing on brain pathology as a function of disease progression.

Additionally, as reviewed in Amaral, Schumann, & Nordahl (2008), human imaging and animal lesion studies (Adolphs, 2001) have found abnormalities in a wide range of brain areas related to social functioning and communication processes. These include the orbitofrontal cortex, amygdala, caudate nucleus, Broca's area, Wernicke's area and the hippocampus. Results have suggested that ASD involves dramatic changes in dendritic growth (Rockland & Ichinohe, 2004), neuronal loss (Kemper & Bauman, 1993), laminar organization (Casanova, Buxhoeveden, Switala, & Roy, 2002) and glial populations (Pardo, Vargas, & Zimmerman, 2005). Although it may seem that every brain region has been implicated in autism, there are discrepancies across techniques and brain areas involved are likely to vary across subpopulations of patients. Even so, findings of developmental changes with an initial general increase in overall white matter, followed by retarded growth, have generally been agreed upon (Blaylock, 2008; Hendry et al., 2006; Herbert et al., 2003).

1.3 ASD as a Whole Body Disorder

Because of all of the abnormalities noted in neuronal and cell cytoarchitecture, ASD has historically been presented as a strictly neurological disorder where the cognitive and behavioural abnormalities arise specifically from the central nervous system (CNS). This has been called into question however, with the observation that many children with ASD also display gastrointestinal discomfort, inflammatory bowel syndrome, constipation, diarrhea, gastroesophageal reflux, colic, food intolerance and food allergies (Horvath & Perman, 2002). One of the more pressing issues in ASD populations is that GI discomfort frequently leads to bouts of problem behaviours including unusual irritation, aggression, the onset of self-injury and an overall non-compliance of demands (Buie et al., 2010). Coury et al. (2012) speculated that underlying mechanisms relating to these gut related

problems are most likely a combination of changes in gut flora, intestinal permeability and inappropriate immune responses. Results of a variety of animal experiments have provided evidence for the GI tract's extensive influences on neurotransmitter synthesis, immune markers, hormone production and metabolism (Bienenstock, Forsythe, Karimi, & Kunze, 2010; Gonzalez et al., 2011; Jyonouchi, Sun, & Itokazu, 2002). This has contributed to the now prominent view of ASD as a whole body disorder. In this respect, ASD has been suggested as 1) a metabolic disorder and 2) an immune disorder (Frye, Melnyk, & MacFabe, 2013; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005).

1.3.1 ASD as a Metabolic Disorder?

In a subpopulation of ASD patients, mitochondrial dysfunction may play a critical function in its etiology. Thirteen out of the 100 proteins required for ATP production are encoded by the mitochondrial DNA (mtDNA; Dhillon, Hellings, & Butler, 2011) and any abnormalities there can lead to severe complications in energy metabolism. Lactic acidosis, decreased glucose utilization, reduced ATP levels, carnitine deficiency and increased oxidative stress have all been reported in ASD patients (Palmieri & Persico, 2010). In a small sample of ASD children, specific mtDNA deletions have been noted with the patients showing lactic acidosis, developmental delay and seizures (Pons et al., 2004). Likewise, Graf et al. (2000) presented a case study where the child displayed hyperactivity, regression at 2 and fine motor dyspraxia. These specific mutations, however, have only been shown in a very small number of patients, and only 23% of children with ASD have a known mtDNA abnormality (Rossignol & Frye, 2012). Therefore the relationship between ASD and mitochondrial abnormalities are typically described as general energy metabolism deficits rather than a specific genetic deletion (Lombard, 1998).

In light of the findings of alterations in energy metabolism, several investigators have put forth the idea that autism is a disorder of lipid metabolism (fatty acids and membrane phospholipids), highlighting the likelihood that altered homeostasis is a result of dietary factors, inflammation and/or infection (Tamiji & Crawford, 2010). Polyunsaturated fatty acids (PUFAs) of which arachidonic acid (AA), eicosapentaenoic acid (EPA) and

docosahexaenoic acid (DHA) are prevalent, comprise the cell membrane and the results of several studies show that abnormal ratios of these PUFA metabolites are associated with neuropsychiatric disorders such as Attention-deficit/Hyperactivity disorder (ADHD; Richardson & Ross, 2000), schizophrenia (Khan et al., 2002) and bipolar disorder (Schuchardt, Huss, Stauss-Grabo, & Hahn, 2010). In ASD patients, low levels of omega-3 linolenic acid (DHA) and a high omega-6 linoleic acid (AA) to omega-3 ratio have been detected in the plasma (Bell et al., 2010). Interestingly, researchers noted that hyperactivity in children subsided with the administration of omega-3 fatty acids (Amminger et al., 2007). Similarly, carnitine, which is essential for the transport of PUFA into the mitochondria for oxidation and energy production, may be deficient in ASD. In a study conducted by Mostafa, El-Gamal, El-Wakkad, El-Shorbagy, & Hamza (2005), ASD patients had decreased serum free carnitine and elevated plasma lactate levels, which could be indicative of dysfunction in beta-oxidation. Similarly, carnitine esters in the form of acylcarnitines have been shown to be elevated in children with ASD. More specifically, Frye et al. (2013) found that 17% of the large cohort they evaluated had elevated short and long-chain acylcarnitines. Although there are a number of hypotheses pertaining to the mechanisms underlying ASD deficits, based on the previous findings, mitochondrial dysfunction and abnormal fatty acid metabolism are probably involved.

1.3.2 ASD as an Immune Disorder?

Children with ASD have also been found to have numerous aberrant immune processes with a substantial proportion of studies reporting a relationship between immune dysregulation and ASD (Rossignol & Frye, 2012). Furthermore, candidate genes previously linked to ASD have been shown to have actions at the level of immune signaling (Ziats & Rennert, 2011). Increased proinflammatory cytokines (e.g., IL-6, Interferon- γ) have been identified in serum and cerebrospinal fluid (Ciaranello & Ciaranello, 1995), as well there is evidence of adaptive and innate immunity dysfunction. Specific to the CNS, Vargas et al. (2005) examined neuroglial reactions and cytokine expression in post-mortem brain tissue of ASD patients. Astrocytes and microglia, two important components of the innate immune system, were found to be activated in the medial frontal gyrus, the anterior cingulate gyrus and the cerebellum. These findings of increased astrocytic processes and microglial proliferation coincided with elevated levels

of macrophage chemoattractant protein-1 (MCP-1) and thymus activation-regulated chemokine (TARC) irrespective of clinical profile. This is suggestive of a more chronic neuroinflammatory response in ASD patients, similar to that seen in neurodegenerative disorders such as Amyotrophic Lateral Sclerosis and Alzheimer's Disease (Eikelenboom et al., 2002). Although Vargas et al. (2005) did not find any indication of adaptive immunity responses, as judged by the lack of infiltration of T- and B-lymphocytes, other researchers have noted antibody abnormalities. These researchers suggest that maternal autoantibodies may be a causal mechanism in CNS dysfunction. Martin et al. (2008) presented data obtained from introducing abnormal IgG antibodies purified from mothers of autistic children into pregnant rhesus monkeys. It was found that there was a significant change in the offspring's motor behaviour with evidence of increased whole body stereotypies and hyperactivity. This is consistent with the growing evidence linking immune dysfunction with CNS and behavioural disorders (Patterson, 2011; Brynskikh, Warren, Zhu, & Kipnis, 2008)

Depino (2013) has suggested a number of pathways in which immune effects occur, and proposed that the central influence on chronic inflammation is through glial cells. For example environmental factors can induce chronic neuroinflammation, with prominent activation of microglia and astrocytes. Subsequently, activated innate immune molecules can alter adaptive immune cells (e.g. T and B cells), and neurons. Alternatively, peripheral immune molecules, such as immunoglobulins or cytokines, could directly activate glial cells and consequently neurons. Or finally, neurons could be directly impacted by an environmental trigger, thereby inducing glial cell activation and the recruitment of peripheral immune molecules. These pathways may be of central importance in ASD and are directly pertinent to the current studies.

1.3.3 Gastrointestinal Link

It is unlikely that metabolics and immunity are separate factors in ASD, however, and it is probable that they both contribute to the etiology and represent an integrated system with a reciprocal relationship (Palmieri & Persico, 2010). For example nitric oxide, which is a potent product of oxidative stress (Davis & Williams, 2012) and a neurosignalling molecule (Endoh, Maiese, Pulsinelli, & Wagner, 1993), is important to both. A possible

biological system that links these two are the gastrointestinal microbiota, encompassing specific bacterial populations and the microbiome, referring to the underlying gut ecosystem. The intestinal tract is colonized with approximately 10^{14} bacterial cells (Whitman, Coleman, & Wiebe, 1998) and plays a very important role in the maintenance of host homeostasis under normal physiological conditions (Forsythe & Bienenstock, 2010). Gut dysbiosis, defined as alterations in the microbiota with the loss of some colonies and the overgrowth of others (Petrof, Claud, Gloor, & Allen-Vercoe, 2013), is a prominent finding in ASD, suggesting that certain bacterial species may have a causative or correlational relation to ASD symptomology. Numerous microbial genera have been implicated in ASD, including *Desulfovibrio*, *Bacteriodes* and *Clostridium* (Finegold et al., 2002; 2010; Parracho, Bingham, Gibson, & McCartney, 2005). Interestingly, the microbiota have similarities within families with ASD children (Gonzalez et al., 2011), which could circumstantially explain the heritability seen in ASD diagnoses. Finegold et al. (2010) found that the microbiota present in ASD patients were statistically similar to the microbiota of their siblings with there being evidence of decreased quantities in the phylum *Firmicutes* and reduced levels of genus-specific *Bifidobacterium*. Interestingly *Bifidobacterium* are important probiotics; organisms that promote health effects (Forsythe & Bienenstock, 2010). Therefore not only are the harmful bacteria increased, but the homeostatic species are decreased. Repeated antibiotic usage may be an important risk factor for permanent changes in the microbiota (Looft & Allen, 2012) and ASD children have reportedly high exposure to these compounds (Atladottir et al., 2010). These results, along with the fact that the microbiome is rapidly developing, and thus plastic until three years of age (Koenig et al., 2011), implicates the gut and its components as an avenue worth examining.

There are now extensive data indicating that gut bacteria and their products modulate neurotransmission, metabolic regulation, immune activity and endocrine control (Bienenstock et al., 2010). For example, *Lactobacilli*, a bacterial species common to the gut, have the ability to decarboxylate glutamate for GABA, a compound that can act both centrally and peripherally (Higuchi, Hayashi, & Abe, 1997). Correspondingly, researchers have shown that under stress, epinephrine and norepinephrine can directly bind to

receptors on bacterial species, such as *Escherichia Coli*, and promote neurotoxic effects (Freestone, Sandrini, Haigh, & Lyte, 2008).

1.4 Animal Models of ASD

Several animal models of ASD have been suggested and these range from evaluating pre- or perinatal exposure to chemicals using organophosphates (Bouchard, Bellinger, Wright, & Weisskopf, 2010), valproate (Ingram, Peckham, Tisdale, & Rodier, 2000), ethanol (Arndt, Stodgell, & Rodier, 2005) and thalidomide (Narita et al., 2002), to investigations of the roles of early pre- and postnatal infections (e.g. Borna Virus (Lancaster, Dietz, Moran, & Pletnikov, 2007), Rubella (Chess, Fernandez, & Korn, 1978), and *T. gondii* (Prandota, 2010)). Additionally animal models have been developed using Lipopolysaccharide to emulate the effects of prenatal bacterial infection (LPS; Golan, Lev, Hallak, Sorokin, & Huleihel, 2005) and Poly-IC to mimic prenatal viral infection (Malkova, Yu, Hsiao, Moore, & Patterson, 2012) to assess the consequences of maternal immune system activation.

One of the first steps in evaluating these animal models is to compare the findings against the common features of ASD. The most reported of these pertain to behavioural changes with rodents being assessed for the 3 core deficits in ASD: communication deficits, disinterest in social interaction and stereotypical motor movements (DSM-IV-R, 2000). Therefore researchers have analyzed rodents' behaviour for neophobia: fear of new experiences, perseveration, anxiety, object fixation, hyperactivity, ultrasonic vocalizations, self-grooming rituals and sensorimotor-gating through pre-pulse inhibition (Patterson, 2011). Lucchina, Carola, Pitossi, & Depino (2010) for example, found that injecting mice with LPS on postnatal day 3 resulted in increased anxiety in adulthood. Similarly mice treated with LPS on postnatal day 5 showed an increased startle response in adulthood (Granger, Hood, Ikeda, Reed, & Block, 1996). In rats, early postnatal exposure led to memory impairments (Bilbo et al., 2005). These findings suggest that immune responses to postnatal bacterial infections can adversely affect behaviour. There has yet to be a model that reproduces all of the behavioural components of ASD. This suggests the likelihood of a variety of underlying causal factors.

1.5 Possible Involvement of Enteric Short Chain Fatty Acids

The diversity of the microbiota is frequently cited as being important to host health (Bienenstock et al., 2010) and thus studies where the microbiota are altered, such as in germ-free animals, allow the relationship between the microbiota, metabolism, and the immune system to be elucidated. Vijay-Kumar et al. (2010) have shown that mice reared without Toll-Like Receptor 5 (TLR-5), an immune receptor present on the gut mucosa, have altered microbiota and display altered eating behaviour and evidence of a metabolic syndrome. When germ-free mice were then colonized with the microbiome lacking TLR-5, the associated phenotype appeared in these rodents. The researchers thus inferred that the changes in bacterial composition directly altered the eating behaviour through an innate immunity mechanism. Overall however, the specific mechanisms by which bacterial species alter metabolism and immune function in the central and enteric nervous systems to produce ASD and GI symptoms are largely unknown. It has recently been proposed that propionic acid (PPA), a short chain fatty acid that is a metabolic byproduct of enteric bacteria, an intermediary of fatty acid metabolism and a food preservative (Thompson et al., 1990) may be a putative factor for ASD (MacFabe, 2012).

A number of researchers have focused on characterizing the effects of PPA both systemically and centrally and have described several consequences of elevated PPA levels. In the gut, PPA can activate immune effector molecules (e.g., mast cells) (Karaki et al., 2006) and neurotransmitters (e.g. serotonin; Mitsui, Ono, Karaki, & Kuwahara, 2005), either directly through its own receptors or indirectly. In the CNS, PPA has been shown to affect NMDA receptor activity (de Mattos-Dutra et al., 2000), intracellular calcium levels (Nakao, Fujii, & Niederman, 1992), gap junction gating (Rorig, Klaus, & Sutor, 1996), oxidative phosphorylation (Brass & Beyerinck, 1988), carnitine availability (Brass & Beyerinck, 1987), cytokine release (Cavaglieri et al., 2003), and the expression of a variety of genes. All of these deficits have been shown in ASD patients (see MacFabe, 2012 for a review), supporting PPA elevations as a possible, important mechanism in psychiatric disorders. It is important to note, however that PPA is not intrinsically negative and studies have shown beneficial effects with the fermentation of undigested carbohydrates mediating cholesterol levels and decreasing appetite, albeit through slow gastric emptying (Arora, Sharma, & Frost, 2011; Wolever, Fernandes, &

Rao, 1996) and/or internal aversive cues (Ossenkopp et al., 2012). It is also likely that enteric bacteria can produce increased amounts of PPA thereby modifying gut contraction and motility (Cuhe & Malbert, 1999) leading to gastrointestinal dysfunction and neurological symptoms.

In light of these findings, PPA has been implicated in Propionic acidemia (Brusque et al., 1999), now ASD (MacFabe et al., 2007) and its related compound 3-nitropropionic acid, in Huntington's Disease (Borlongan et al., 1997). Propionic acidemia is categorized as one of many inborn errors of metabolism, and is an autosomal recessive disorder resulting from a deficiency of propionyl-CoA carboxylase enzyme. This deficiency causes increased amounts of propionyl-CoA to be converted to PPA, resulting in general elevations in several metabolites (Brusque et al., 1999). Behaviourally these patients have symptoms such as irritability, hyperactivity, and stereotypic movements: lip smacking, tongue thrusting, rhythmic jerking and posturing (Burlina, Bonafe, & Zacchello, 1999). Metabolically, carnitine deficiency is also common in these patients and supplementation has been proposed as a therapeutic treatment (El-Ansary, Shaker, El-Gezeery, & Al-Ayadhi, 2013). In a study performed by Surtees, Matthews, & Leonard (1992), neurological outcomes were addressed in two groups of Propionic acidemia patients: early onset diagnosed within the first week of life, and late onset diagnosed 6 weeks after birth. They found that the children diagnosed neonatally displayed severe mental deficits, whereas those diagnosed later were more likely to have a mild to moderate movement disorders. Despite differences in clinical manifestation however, there was no difference in severity of the enzyme deficiency such that both groups had similar PPA levels. It is therefore possible that there is a timing of exposure effect in which elevated PPA is particularly damaging. This is further shown in a study by Brusque et al. (1999) who characterized the pharmacokinetics of PPA injected systemically in young rodents. They discovered that the brain had a critical period of vulnerability that decreased with age. More specifically because the blood-brain barrier is more permeable at a younger age, there was increased PPA levels in the CNS in young compared to mature rodents. Not only does this coincide with findings of inborn metabolism errors being the most damaging neonatally, but this could indicate that there is a specific time frame in which PPA exposure could contribute to ASD neuropathology. Interestingly, a case study

reported by Al-Owain et al. (2013) found that a child diagnosed with Propionic acidemia at birth met all of the diagnostic criteria for autism by age 3. It can be inferred that as a result of the metabolic enzyme deficiency, high levels of plasma PPA were able to cross the blood-brain barrier through either active (Bergersen, Rafiki, & Ottersen, 2002; Nagasawa et al., 2006) or passive transport (Karuri, Dobrowsky, & Tannock, 1993) and induce the triad of symptoms associated with ASD.

1.5.1 PPA Animal Model

A necessary component of establishing an animal model is the presence of face and construct validity (van der Staay, 2006). Through the results of experiments using various dosing regimes, evaluating different behavioural parameters and quantifying neuropathological and lipid changes, it is postulated that this has been ascertained and that the PPA model can provide insight into the role of gastrointestinal and dietary factors in the etiology of ASD. Using a high dose (0.26M) of PPA and a low dose (0.052M) of PPA, MacFabe et al. (2007) examined the electrophysiological, behavioural and neuropathological effects of centrally infused PPA in rats delivered through an intracerebroventricular (ICV) cannula. Importantly not only were ASD-like behaviours present (e.g., hyperactivity, stereotypic motor behaviours with retropulsion, turning and limb dystonia), but there was evidence of an innate inflammatory response in the hippocampus and the white matter of the external capsule. Specifically, Cluster of Differentiation 68 (CD68), an antibody that marks surface proteins of monocytes and macrophages was used to demonstrate the proliferation of activated microglia, and Glial Fibrillary Acidic Protein (GFAP), a marker that is abundantly produced during cell injury, was used to visualize hypertrophic activated astrocytes. These findings led to the characterization of innate inflammation in conjunction with PPA-induced social and cognitive deficits. Rodents infused with PPA once a week for two weeks (Shultz et al., 2008) demonstrated decreased play interactions, a greater mean distance apart and reduced time spent in the proximity of another rat. Immunohistochemical analyses revealed astrogliosis (GFAP) in the hippocampus following the second infusion. Similarly, MacFabe, Cain, Boon, Ossenkopp, & Cain (2011) found that adolescent rats centrally infused with PPA preferred interacting with objects compared to matched rodent controls. Furthermore, there was evidence of astrocytic (GFAP) and microglial activation

(CD68) in the hippocampus and white matter. Shultz et al. (2009) also presented results suggesting deficits in cognitive tasks with significant perseveration in learning.

Biochemical studies revealed that PPA treated animals have decreased glutathione (GSH) levels in conjunction with increased lipid peroxidation and protein carbonylation (MacFabe et al., 2008). Taken together these findings are indicative of an increase in oxidative stress in discrete brain regions which can be interpreted as resulting from PPA's reported effects on energy metabolism. Further evidence of deficits in metabolism were revealed in that the PPA group presented with increased levels of short- and long-chain acylcarnitines, decreased unbound carnitine and unusual proportions of saturated and unsaturated fatty acids (Thomas et al., 2010; 2012). These findings have been replicated in human ASD patients suggesting possible predictive validity for the PPA-model (Frye et al., 2013).

1.6 Proposed Research

The aim of the current series of experiments was to examine temporal changes in locomotor activity and neuroinflammation occurring after 1) a single central infusion of PPA, and 2) four spaced central infusions. Prior studies have focused on examining the central and systemic effects of multiple exposures to PPA. This work has evaluated behaviours characteristic of ASD such as anxiety, cognitive dysfunction, hyper-reactivity to sensory stimulation, lack of social interest and movement disorders. Whether these behaviours occur within a particular time frame, however is unknown (MacFabe et al., 2011; Shultz et al., 2008; 2009). Similarly, although markers of astrocytes and microglia have been shown to be elevated in the brains of PPA treated animals, the time course of this activation has not been determined. By giving a single ICV treatment of PPA (Chapter 2) and repeated ICV infusions of PPA (Chapter 3), and perfusing animals at various time points thereafter, the relationships between hyperactivity, anxiety-like behaviour and innate inflammation in the hippocampus (CA1-CA2 and CA3/Dentate Gyrus) and adjacent white matter can be established. In terms of behaviour, rodents were evaluated for total distance traveled, mean velocity and total duration spent in the centre of an open field. In terms of neural changes, activated astrocytes (GFAP) and microglia (CD68) were quantified.

Previously, researchers have reported that several days following a CNS insult, astrocytes and microglia become significantly activated (Chen, Pickard, & Harris, 2003; Norton, 1999; O'Callaghan, Jensen, & Miller, 1995), and there is reason to expect that in the PPA animal model locomotor activity and neuroinflammation occur along different timelines (MacFabe, 2012). Specifically, locomotor activity has been found to be increased within an hour of PPA administration (Brusque et al., 1999; MacFabe et al., 2007), while neuroinflammatory changes were more protracted. In light of these findings, it was hypothesized that in both the single and repeated acute administrations of PPA, animals will exhibit increased locomotor activity within a short time period followed by the activation of neuroinflammatory markers over a longer time frame of several days.

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Chapter 2

Temporal Changes of Locomotor Behaviour and Neuroinflammation following a Single Intracerebroventricular Infusion of Propionic Acid in rats.

2.1 Introduction

Autism Spectrum Disorders (ASD) are a family of neurologically based disorders that include Autistic Disorder, Asperger's Syndrome, Childhood Disintegrative Disorder and Rett's Disorder (*DSM IV-TR*; American Psychiatric Association, 2000). Although it is by definition a spectrum of disabilities, most of the individuals referred for treatment have abnormalities in three main symptom domains: Social functioning, communication and language processes and behavioural expression. In addition to these core symptoms, ASDs are known to be comorbid with several disorders including epilepsy, mental retardation, gastrointestinal disturbances and anxiety or mood disorders.

Anxiety and mood disorders have commonly been presented as strictly neurological, however, it has been shown that these disorders can be associated with chronic gastrointestinal diseases such as Irritable Bowel Syndrome and Chron's disease (Bienenstock, Forsythe, Karimi, & Kunze, 2010). Similarly, children diagnosed with ASD have reported gastrointestinal disturbances such as constipation, diarrhea, bloating, gastric reflux and food sensitivities (Horvath & Perman, 2002), and researchers have found abnormal colonies of gut bacteria in their stool (Finegold et al., 2010; Parracho, Bingham, Gibson, & McCartney, 2005). The etiology of ASD is largely unknown with only a small subset of patients having clear genetic causes, and recent evidence has suggested that dietary, gastrointestinal and environmental factors may contribute to ASD. It thus remains plausible that alterations in the microbiome of the gut is a putative factor in the etiology of ASD, especially in view of the diverse effects that the gut has on immune system activation, lipid metabolism and central nervous system activity (Forsythe, Sudo, Dinan, Taylor, & Bienenstock, 2010; Gonzalez et al., 2011; Patterson, 2011).

The concept of bacterial agents being able to affect host behaviour is not novel (Gonzalez et al., 2011; Freestone, Sandrini, Haigh, & Lyte, 2008). *Brucella*; gram-negative bacteria have consequences in cognitive and emotional disorders (Eren et al., 2006), *Streptococcus*; gram-positive bacteria in neuropsychiatric disorders like PANDAS (Swedo, Leonard, & Rapoport, 2004), and *Mycobacterium tuberculosis* in anxiety, depression and OCD (Vega et al., 2004). Therefore the evidence that bacterial species in

the gut bear some relation to ASD symptoms is gaining credence. Researchers that have isolated bacterial species present in the gut, have found that stool samples collected from ASD patients contain a significantly higher diversity of bacteria and a 10-fold increase in *Clostridium* species as compared to controls (Finegold et al., 2002; Finegold, 2008). This higher proportion of clostridia was correlated with gastrointestinal problems in ASD patients (Parracho et al., 2005).

Clostridium are a family of anaerobic, spore-forming gram-positive rods that if found in abnormally large proportions can lead to gastrointestinal infections such as *Clostridium difficile* disease. It has been proposed that the ability to form spores may account for the fluctuations in symptomology typical of ASD (Finegold, 2008). Interestingly when Vancomycin, an antibiotic that targets *Clostridium*, was administered to a small sample of ASD children, there were significant improvements in expressive language, receptive language, perseveration, and compliance (Sandler et al., 2000). Similarly the core manifestations of ASD appear to be abated with the introduction of probiotics (Critchfield, van, Ash, Mulder, & Ashwood, 2011) and omega-3s (Amminger et al., 2007), which target the gut microflora and regulate lipid functioning, respectively. These findings indicate that ASD may be a disorder of abnormal fatty acid metabolism. Furthermore, it has been suggested that these microbial metabolites induce an innate immune response (Bienenstock et al., 2010; Jyonouchi, Sun, & Itokazu, 2002) that may have further consequences for the neuropathology that is typical of ASD (Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005).

MacFabe and colleagues (2007; 2008; 2011; Shultz et al., 2008; 2009) have provided further evidence for this idea by inducing autistic-like features in a rat using a metabolic byproduct of enteric bacteria. Propionic acid (PPA) is a short chain fatty acid that is an intermediary of fatty acid metabolism and a common food preservative. It is produced by many bacterial species, with the majority of *Bacteroidetes* being important metabolizers. Interestingly, this phylum was overrepresented in the gut of ASD patients (Finegold et al., 2010). It has been shown that in disorders such as Propionic Acidemia, patients receive relief from GI discomfort through administration of metronidazole, an antibiotic that reduces PPA-producing bacterial populations (Grunert et al., 2013).

In rats, PPA has been shown to have a variety of effects on social behaviour, locomotor activity, object fixation, and spatial learning processes when administered via intraperitoneal (IP; Benzaquen et al., 2010; Ossenkopp et al., 2012) and/or intracerebroventricular (ICV) routes (MacFabe et al., 2007; 2008; 2011; Shultz et al., 2008; 2009). Additionally, the presence of innate inflammatory markers such as activated microglia and astrogliosis in the hippocampus of ICV administered PPA treated rats have been quantified and found to be elevated as compared to controls. Generally, increased expression of Cluster of Differentiation 68 (CD68) corresponds to activated microglia whereas elevated production of Glial Fibrillary Acidic Protein (GFAP) is indicative of reactive astrogliosis. The exact mechanisms by which these neuroinflammatory changes and behavioural alterations occur are largely unknown. However PPA's ability to influence lipid metabolism (Thomas et al., 2010; Thomas et al., 2012), gene expression and immune and mitochondrial functioning (Brass & Beyerinck, 1988), are likely key contributors. Of particular interest in the current study is the presence of innate neuroinflammation, principally reactive astrocytes and activated microglia, in the brain. PPA can be taken up by these glial cells (Maurer, Canis, Kuschinsky, & Duelli, 2004) and through a process of intracellular acidification (Karuri, Dobrowsky, & Tannock, 1993), may compromise their functioning.

The importance of examining activated astrocytes and microglia, and deciphering their rate of activation, lies in their abilities to act as both neuroprotectants and neurotoxins. Under normal physiological conditions, astrocytes are crucial to the survival of neurons in the CNS through their roles in blood brain barrier integrity, glutamate uptake and lactate release, formations of gap junctions for neuronal spatial buffering, neurotransmitter synthesis and release, and antioxidant metabolism (e.g., glutathione; Chen & Swanson, 2003) (Review by Kirchhoff, Dringen, & Giaume, 2001). Similarly astrocytes possess enzymes for amino acid metabolism and carbohydrate metabolism including pyruvate carboxylase for the synthesis of oxaloacetate (Cesar & Hamprecht, 1995) and glycogen phosphorylase for the mobilization of glycogen (Reinhart, Pfeiffer, Spengler, & Hamprecht, 1990). If astrocytes become damaged, dysfunctional and/or activated, all of these functions are affected and tend to propagate toxic insults resulting in further damage (De Keyser, Mostert, & Koch, 2008). For example in the late stages of activation, glial

scar formation can hinder neuronal regeneration (Sofroniew & Vinters, 2010). Typically, the presence of glial cytoskeletal filaments is indicative of CNS injury, which is why GFAP is a useful marker. The consequences of activating astrocytes are numerous and include: the infiltration of lymphocytes, chemokines and cytokines, the activation of signaling cascades leading to the synthesis of reactive oxygen species (ROS) such as nitric oxide (Endoh, Maiese, Pulsinelli, & Wagner, 1993), and the inhibition of gap junction coupling (Siushansian, Bechberger, Cechetto, Hachinski, & Naus, 2001). All of these increase vulnerability of the surrounding tissues in a cyclic pattern: increased astrogliosis, leads to the synthesis and/or release of adverse chemokines, cytokines and ROS, which in turn induce recruitment and/or activation of immunomolecules. For example some researchers believe that reactive astrocytes recruit microglial cells to the site of injury and directly stimulate their proliferation (Ovanesov et al., 2008).

While the particular changes that astrocytes undergo in order to become reactive are only now being determined, microglial activation has been well characterized. Resident microglia, which are cells of mesodermal origin and thus have the capacity to become phagocytic (Ling & Wong, 1993), undergo three successive changes: 1) Proliferation, 2) Increased expression of immunomolecules such as major immunohistocompatibility complex (MHC) antigens, and 3) Recruitment to site of injury and functional changes, for example the release of inflammatory cytokines (e.g., IL-1, IFN- γ , TNF- α ; Dickson, Lee, Mattiace, Yen, & Brosnan, 1993) and production of ROS (Gehrmann, Matsumoto, & Kreutzberg, 1995). Following these changes, microglial cells are activated but not phagocytic. Once phagocytic, the microglial cells are defined as reactive (Montero-Menei et al., 1996). The circumstances under which reactive microgliosis occurs are not well understood, however the degeneration of neurons appears to be a fundamental condition (Streit & Kreutzberg, 1988). Similar to astrocytes, resident microglia are important to normal CNS function. They regulate the microenvironment to ensure homeostatic conditions (Nimmerjahn, Kirchhoff, & Helmchen, 2005), and have recently been explored as players in synaptic remodeling (Paolicelli et al., 2011). More specifically, when microglia were depleted from the hippocampus, there was a significant increase in the frequency of excitatory postsynaptic currents. This was interpreted as modulation of synaptic activity through the expression of glutamate receptors, especially since once

microglial cells were placed back in culture, there was a decrease in AMPA receptor mediated excitation (Ji, Akgul, Wollmuth, & Tsirka, 2013). In spite of this, microglial cells are typically described as crucial immune activators. It is anticipated that by evaluating the temporal relationship of astrogliosis and activated microglia, the roles of these glial cells will be better understood in PPA-induced neuroinflammation.

In prior studies with rats, the PPA dosing paradigm has utilized a chronic schedule with infusions administered twice a day for seven or fourteen days, once a week for 5 weeks or once a day for 5 days (MacFabe, 2012). The objective of the current study is to evaluate the effects on locomotor activity and glial cell activation associated with a single ICV infusion of PPA. Previously there have been inconsistent findings in regards to PPA-induced hyperactivity and anxiety. Specifically, MacFabe et al. (2007; 2008) reported frequent incidences of increased locomotor activity in PPA treated animals, while Shultz et al. (2008) found no significant differences in activity and Ossenkopp et al. (2012) provided evidence for hypoactivity in systemically injected animals. By investigating the locomotor behaviour associated with a single infusion, the specific consequences on open field activity can be determined. In particular activity parameters such as total distance traveled, mean velocity and total duration in various zones, will be measured immediately following ICV infusion. The latter will provide a measure of thigmotaxis, which can be used as an index of anxiety (Treit & Fundytus, 1988).

In addition to deciphering PPA's effect on locomotor activity, by perfusing animals 30 minutes, 1 hour, 24 hours, 48 hours or 72 hours following injection, the effects of ICV PPA on astrocyte and microglia function will be assessed. Immunohistochemical analyses will involve the quantification of activated astrocytes (GFAP) and microglia (CD68) in the dentate gyrus, hippocampus and adjacent white matter. As previously reviewed, the innate inflammatory system becomes activated along a later timeline and thus it is hypothesized that if a single injection is sufficient to activate astrocytes and/or microglia, the reaction will occur within several days.

2.2 Methods

2.2.1 Subjects

A total of 80 adult male Long-Evans rats were used (Charles River Laboratories, Quebec, Canada). At the time of surgery, rats weighed 250-350g and were housed in pairs in standard polypropylene cages in a colony room with a controlled ambient temperature of $21 \pm 1^\circ\text{C}$. Following surgical implantation of a cannula, the animals were housed individually and permitted 14 days to recover. The colony room was kept at a 12:12 hour light:dark cycle and all behavioural testing was conducted during the light period from 0700h to 1900h. The animals were given *ad libitum* access to food (ProLab rat chow) and tap water and were naïve to all experimental procedures. Housing and testing protocol were performed in accordance with the guidelines set out by the Canadian Council for Animal Care (CCAC) and were approved by the University of Western Ontario Animal Use Subcommittee (see Appendix A).

2.2.2 Surgical Implantation of Guide Cannula

All of the rats were implanted with a 23-gauge guide cannula under standard stereotaxic procedures. First rats were placed in a Plexiglas box where a 4% isoflurane and 2L/min oxygen flow allowed for the induction of anesthesia. Upon reaching an adequate depth the rats were removed from the box and placed into a stereotaxic device equipped with a nose cover that permitted gas flow during surgery. The animals were then subcutaneously injected with an analgesic (Ketoprofen, 1mL/kg). Under aseptic conditions, a cannula was implanted into the right lateral ventricle with the tip being placed according to the following coordinates in relation to Bregma: -1.4 anterior/posterior, +1.8 medial/lateral and -3.0 dorsal/ventral (Paxinos, Watson, Pennisi, & Toppo, 1985). Stainless steel screws were inserted into the skull to serve as anchors for the adherence of dental acrylic to the cannula and skull. A plastic removable plug was placed in the top of the cannula in order to avoid contamination and silk sutures were used to aid in the healing process.

2.2.3 Treatment Groups

Following two weeks of recovery, rats were randomly assigned to two equal treatment groups (N = 40 for each group) and received a single intracerebroventricular (ICV) infusion of one of two compounds: 4 μL of a 0.26M solution of physiologically-buffered Propionic Acid (PPA, pH = 7.5) or 4 μL of a 0.1M solution of Phosphate Buffered Saline (PBS, pH = 7.5). The drug and saline were administered to the lateral ventricle via a 30-

gauge injection cannula, thereby protruding 0.5mm beyond the tip of the implanted cannula. The injection cannula was connected to a Sage syringe pump by PE10 tubing and the delivery of 4 μ L of solution took approximately one minute to complete. The previously mentioned dose was chosen based on dose-response findings by MacFabe et al. (2007). Each animal received a single infusion immediately (0-45 seconds) prior to behavioural testing.

2.2.4 Behavioural Apparatus

Locomotor activity was evaluated in a circular open field measuring 90cm in diameter and 40cm in height. The floor of the apparatus was covered with Beta chip bedding used to facilitate the contrast between the Long-Evans rat and the surrounding arena. A CD camera and darkroom lamp were located above the centre of the apparatus and the camera was connected to a computer that used *EthoVision 3.0.15 Behavioural Monitoring and Analysis System*, to quantify measures of interest. This computer program was capable of tracking the x-y coordinates of the animal and was therefore used to compute 1) total distance traveled (cm) and 2) average velocity (cm/s). Furthermore, the open field was divided into two separate zones with a centre zone equating to the interior 2/3 of the total field, and a peripheral zone equating to the exterior 1/3. This distinction was used to decipher 3) the time spent in the centre zone (s), in order to measure thigmotaxis, an index of anxiety (Treit & Fundytus, 1988). Additionally, the CD camera was connected to a VCR, which allowed for future analysis of behavioural variables.

2.2.5 Experimental Procedure (see Fig. 2.1)

In addition to being randomly assigned to a drug group, each animal was assigned to a group with a time period dictating the interval between their infusion and the administration of a near-lethal dose of sodium pentobarbital (approximately 0.7mL). In this way a timeline of PPA-induced behaviour and neuropathology could be established. The chosen time points were as follows: 30 minutes, 1 hour, 24 hours, 48 hours and 72 hours. Overall there were 10 treatment groups: PPA-infused rats perfused at 30 minutes (N = 8), 1 hour (N = 8), 24 hours (N = 8), 48 hours (N = 8) and 72 hours (N = 8), and their associated PBS controls (N = 8 for all control groups) (Step 1 in Fig. 2.1).

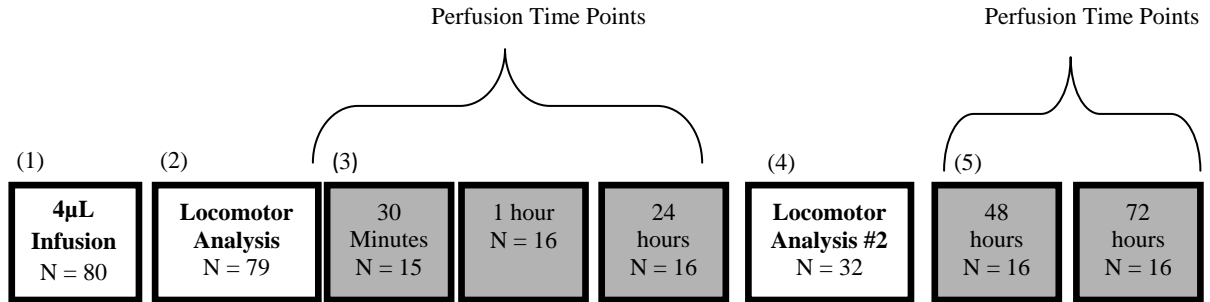


Figure 2.1: Methods Procedure, where N = number of animals. All animals received an ICV infusion into the right lateral ventricle and were immediately placed into the open field for 20 minutes. Behavioural parameters (total distance traveled, mean velocity, and duration spent in centre zone) were computed for all of the groups and then animals were perfused based on time interval. Note that there was a malfunction with the tracking device and one of the rats assigned to the PBS treatment and 30 minute time interval was not analyzed (N = 79 for locomotor analysis, however the brain tissue was still used in the analysis). Perfusion time points refer to the time between ICV injection and receipt of anesthetic dose of euthanyl. Brains were then removed for further analysis.

Once the assigned drug was administered, each rat was placed in the open field apparatus for 20 minutes (Step 2 in Fig. 1). Locomotor activity was monitored for this amount of time based on a pilot study by MacFabe et al. (2007) that provided evidence for the behavioural effects of PPA obtaining maximal values within the first 30 minutes post injection. Additionally the half-life of PPA is known to be between 18 and 57 minutes (Brusque et al., 1999) further supporting this time frame.

A novel method implemented in this study involved the 48-hour and 72-hour groups having their locomotor behaviour measured twice: once immediately following infusion, and again immediately preceding perfusion (Step 4 in Fig. 2.1). This procedure was used to determine whether any evident behaviours returned to baseline values after two or three drug-free days

2.2.6 Brain Tissue Preparation and Histological Procedures

At their assigned time interval, rats were deeply anesthetized with sodium pentobarbital (270mg/mL IP) and were transcardially perfused with 200mL of 0.1M ice cold PBS and 300mL of 4% paraformaldehyde in PBS. The brains were removed, placed in paraformaldehyde for further fixation and stored at 4°C for 24 hours. Following complete fixation, the brains (N = 79 due to incomplete fixation of one PPA-infused 30 minute rat) were transferred into an 18% sucrose solution for cryoprotection. All of the brains were then blocked into 5 equal 2mm sections, defatted and dehydrated through increasing concentrations of ethanol and embedded in paraffin wax. Cannula placement was ascertained visually once brains were blocked. Using a Leica microtome (model RM2125), coronal sections 4µm thick were sliced through the dorsal hippocampus (CA1-CA3 and the hilus of the dentate gyrus) and adjacent white matter (Paxinos & Watson, 1985), and mounted on glass slides (SurgiPath, Canada). The tissues were rehydrated and deparaffinized with xylene and decreasing concentrations of ethanol. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in distilled water.

The following markers were used to determine the presence of innate inflammation: 1) Anti-glial fibrillary acidic protein (GFAP: 1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark) and 2) Anti-rat CD68 antigen (1:200, monoclonal, Serotec, Oxford, UK). Note that antigen retrieval, in which aldehydes were removed through the

immersion of tissues in 0.21% citric acid buffer (pH = 6.0) for 30 minutes, was necessary only in preparation for CD68 antibody binding. After immersion, slides were counterstained with Gill hematoxylin (EMD Chemicals, New Jersey), dipped in acid alcohol, ammonia, and rinsed with distilled water. In anticipation of antibody exposure, tissues were coated with a PBS + 10% horse serum solution. Horse serum, an inert protein, was used to prevent non-specific antibody binding as a consequence of charge differential. Primary antibody (GFAP and CD68) was applied and slides were incubated for 1 hour at room temperature. Following incubation, brain tissues were rinsed with PBS, and the secondary antibody, either biotinylated anti-rabbit (GFAP 1:200, Vector Laboratories, California) or biotinylated anti-mouse (CD68: 1:200, Vector Laboratories, California) was applied for 30 minutes. Again, tissues were rinsed and then stained with an avidin-biotin complex (Vectastain Elite ABC, Vector Laboratories, California) for another 30 minutes. After this final incubation, tissues were washed with PBS, and 3,3'-diaminobenzidine DAB chromagen (Sigma-Aldrich, Missouri) was applied for 5 minutes enabling the markers to be visualized. This histological procedure was completed once the tissues were dehydrated with ascending concentrations of alcohol and coverslipped.

2.2.6.1 Immunohistochemical Quantification

The imaging analysis was used according to the procedure described in previous studies (MacFabe et al., 2007, 2008; Shultz et al., 2008). It involved using a standard light microscope and taking 4 digital pictures spanning the pyramidal layer of the hippocampus from CA1 to CA2, and 4 pictures from CA3 to the hilus of the dentate gyrus. In addition 7 pictures were taken of the adjacent white matter for a total imaging area of 320,000 μm^2 in the right hemisphere (see Fig. 2.2). This pattern was applied to the imaging of each rat brain (N = 77 for GFAP and N = 78 for CD68 due to incorrect tissue preparation) and a set of color recognition criteria was created to evaluate the staining. The wavelengths of red, blue and green were chosen based on their ability to capture the immunopositive staining of each individual image and was different for each antibody to control for the variance in intensity of DAB labeling. Using ImagePro Plus software, the quantification of the staining was conducted for each brain region (CA3-dentate gyrus, CA1-CA2, and white matter). Based on the color recognition criteria, an algorithm was applied where the amount of positive staining was calculated.

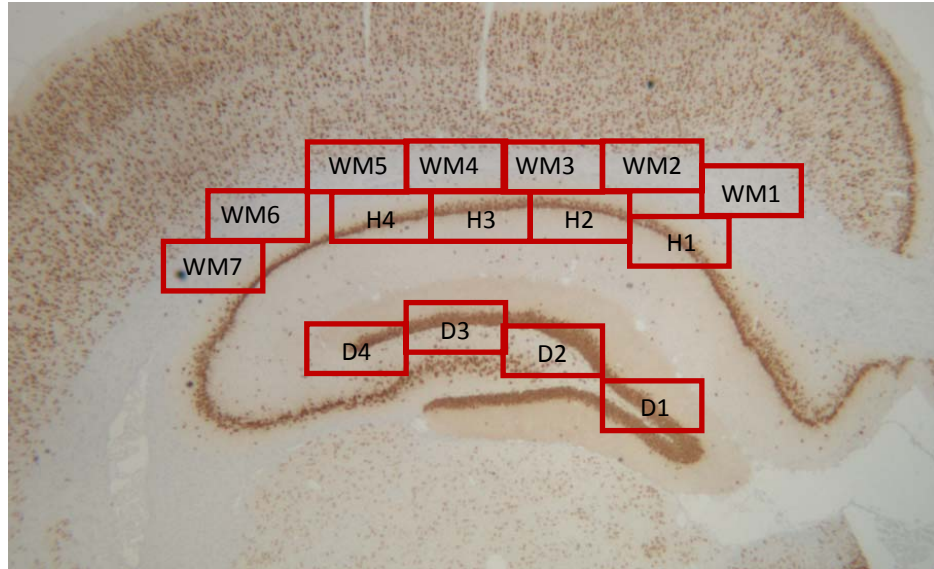


Figure 2.2: Cartoon Image of Coronal Section of Ipsilateral Dorsal Hippocampus indicating the location of the acquired images through the hippocampus and white matter. H1-H4 represents images spanning CA1-CA2, D1-D4 represents images spanning CA3-Dentate Gyrus and WM1-WM7 represents images spanning the white matter of the external capsule. Size of images are not to scale (see section 2.2.6 for actual dimensions)

To evaluate reactive astrocytes (GFAP), each image was measured for its degree of total immunopositive staining per area (μm^2), and the results of each image were summed to produce a total area in each brain region (see Fig. 2.6D for a subset of these images). Because activated microglia are measured by the degree of proliferation, while astrocyte activation is measured by hypertrophic somas (Dihné, 2001), each image stained with CD68 yielded a total cell count. Again the results of each image were summed to produce total count per brain region. See Figure 2.7D for a sample of these images.

2.2.7 Statistical Analysis

To determine whether the effect of PPA on behaviour and brain pathology differed from the control infusion, several analyses were conducted. A one-way analysis of variance (ANOVA) was carried out for the EthoVision activity data with drug treatment (2 levels: PPA and PBS) as the between-subjects factor. It was unnecessary to consider time interval for this particular analysis because all of the animals received their testing at the same time. Additionally, a Repeated Measures design was used to evaluate the return to baseline of behaviour in the 48 hour and 72 hour groups, separately. In this case, Session (2 levels: #1 and #2), with #2 being either 48 or 72 hours of drug-free time, was the within subjects variable whereas drug (2 levels: PBS and PPA) was the between-subjects variable. A 2 x 5 ANOVA was also conducted for GFAP and CD68 in the dentate gyrus (CA3-DG), hippocampus (CA1-CA2), and white matter. Drug treatment (2 levels: PPA and PBS) and perfusion time (5 levels: 30 minutes, 1 hour, 24 hours, 48 hours and 72 hours) were the between subjects factors. Multivariate ANOVAs (MANOVA) were used to analyze brain regions across the 10 treatment groups and Tukey's HSD post-hoc analysis were conducted where appropriate. An alpha level of 0.05 was used as the significance criterion in all of these analyses.

2.3 Results

2.3.1 Behaviour

2.3.1.1 Hyperactivity Measures

The first measure to assess hyperactivity, total distance traveled, resulted in a significant main effect of drug ($F(1,77) = 25.69, p < 0.001$) with the PPA-infused animals moving a greater overall distance than the PBS-infused animals (Fig. 2.3A). Similarly, analysis of

the average velocity exhibited by the rats revealed a significant main effect of drug ($F(1,77) = 25.70, p < 0.001$) with the PPA treated animals traveling at greater speeds than the PBS infused animals (Fig. 2.3B). Overall, the PPA group traveled further and faster, thereby exhibiting the main characteristics of hyperactivity.

To examine whether these locomotor effects returned to baseline values following several days of recovery, ANOVAs were conducted and revealed a significant drug by session interaction for total distance traveled. As depicted in Fig. 2.4A, animals reassessed 48 hours after infusion (interaction: $F(1,14) = 11.38, p < 0.01$) and 72 hours after infusion (interaction: $F(1,14) = 4.78, p < 0.05$) showed values similar to control levels. Post-hoc analyses revealed that the animals that received PPA traveled a significantly greater distance on infusion day than any of the other groups (48 hours: $p < 0.01$, 72 hours: $p < 0.05$). Likewise a significant drug by session interaction was obtained for mean velocity (see Fig. 2.4B) in animals reassessed at 48 hours ($F(1,14) = 11.37, p < 0.01$) and 72 hours ($F(1,14) = 4.67, p < 0.05$) post-infusion. Again post-hoc analyses indicated that animals given PPA moved significantly faster on the infusion day than any of the other groups (48 hours: $p < 0.01$, 72 hours: $p < 0.05$). It can therefore be inferred that PPA-induced hyperactivity was transient.

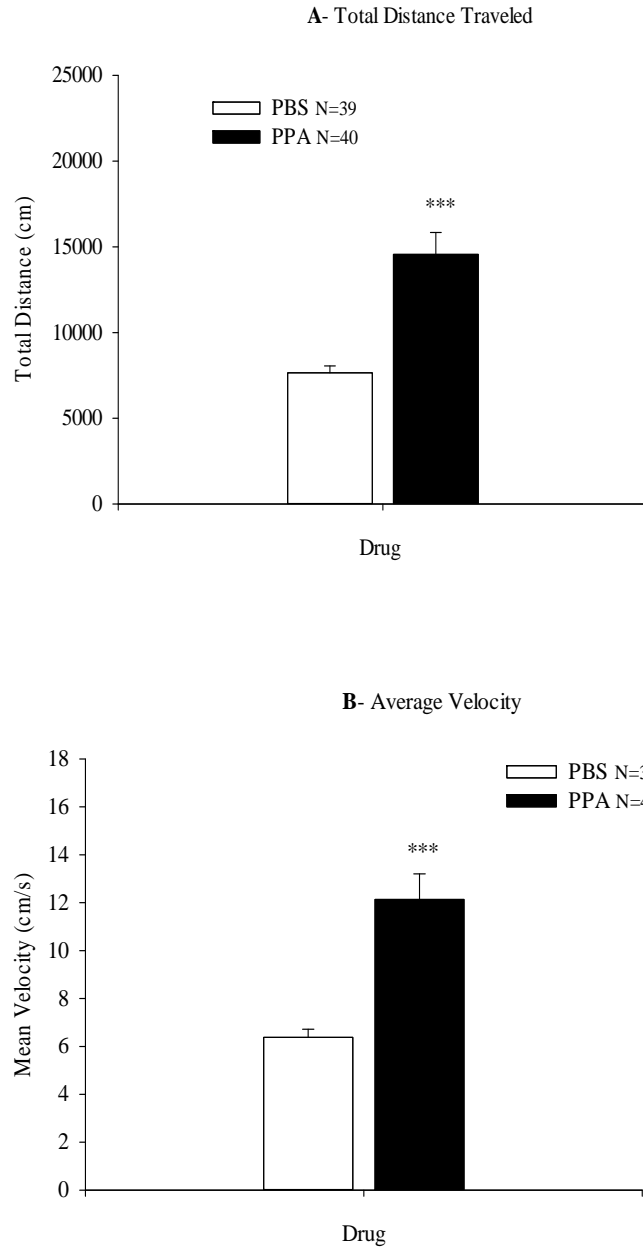


Figure 2.3: Evaluation of locomotor activity with (A) total distance traveled (cm) and (B) average velocity (cm/s) in an open field of animals injected ICV with PBS or PPA. Each bar represents group mean data for the 20 minutes immediately following infusion. Error bars represent +SEM. *** $p < 0.001$, PPA-infused animals significantly greater than PBS-infused animals.

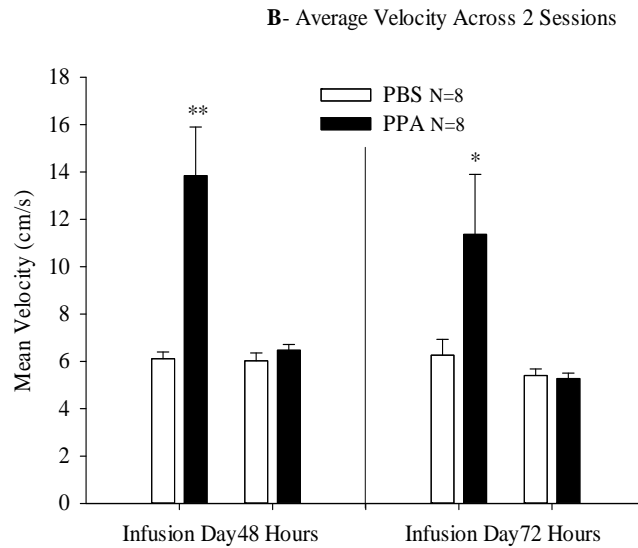
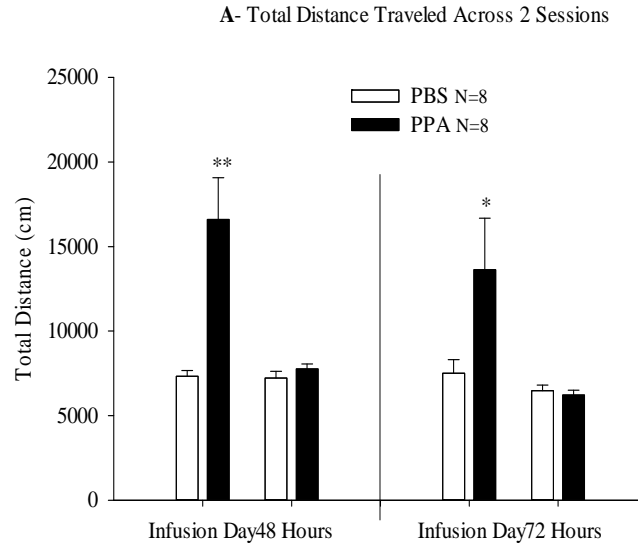


Figure 2.4: Return to baseline analysis in 48 and 72 hour animals with (A) total distance traveled (cm) and (B) mean velocity (cm/s) measured in an open field across animals injected ICV with PBS (each panel N = 8) or PPA (each panel N = 8)..Infusion Day refers to group means of 48 hour animals (Panel #1) and 72 hours (Panel #2) immediately following their single infusion. These means differ from Fig. 2 in that the former is collapsed across time interval. ** $p < 0.01$, PPA treated animals on Infusion Day for 48 hour animals significantly greater than PBS on Infusion Day and PPA/PBS groups 48 hours later. * $p < 0.05$, PPA infused animals on Infusion Day for 72 hour animals significantly greater than PBS on Infusion Day and PPA/PBS treated animals 72 hours later.

2.3.1.2 Thigmotaxis

Thigmotaxis measures anxiety-like behaviours, and the less time spent in the centre of an open field is indicative of anxiety (Treit & Fundytus, 1988). Along these lines, a significant main effect of drug was found ($F(1,77) = 11.30, p < 0.01$) with PPA infused animals spending less time in the centre zone (inner 1/3 of the field) than the PBS groups on the injection day (Fig. 2.5)

Again it was important to determine return to baseline of behaviour following two or more days of no drug. The repeated measures design indicated that there was a session by drug interaction with anxiety-like behaviours in that they did return to baseline within 48 hours ($F(1,14) = 8.89, p < 0.05$), but not at 72 hours ($F(1,14) = 0.86, ns$). The animals that were permitted to recover for 72 hours showed no main effect of drug ($F(1,14) = 1.61, ns$) or session ($F(1,14) = 0.42, ns$). It is possible that the procedure itself is anxiety-inducing considering the variability between sessions appears to be between PBS infused animals. These results are depicted in Figure 2.6.

2.3.2 Neuroinflammatory Response

2.3.2.1 Astrogliosis (GFAP)

The 2 x 5 ANOVA revealed a significant drug by perfusion time interaction for astrogliosis in the dentate gyrus ($F(4,67) = 5.68, p < 0.001$), CA1-CA2 ($F(4,67) = 4.22, p < 0.01$), and adjacent white matter ($F(4,67) = 2.90, p < 0.05$). A MANOVA and Tukey's post-hoc revealed that in all of the analyzed brain regions, the group of PPA treated animals perfused at 48 hours exhibited the greatest increase in immunopositive staining (Fig. 2.7A: $F(9,67) = 9.60, p < 0.001$; Fig. 2.7B: $F(9,67) = 6.06, p < 0.001$; Fig. 2.7C: $F(9,67) = 3.87, p < 0.01$, respectively) with none of the other treatment groups varying across drug or perfusion time. Interestingly by 72 hours this increase in mean area was not different from baseline values. Figure 2.7D represents a qualitative analysis of GFAP staining showing that the astrocytes had become hypertrophic 48 hours following PPA infusion.

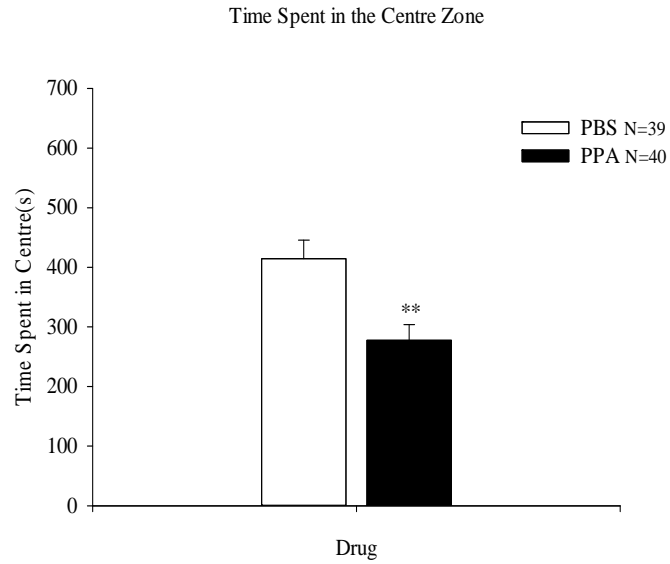


Figure 2.5: Duration spent in the centre zone was evaluated in an open field for 20 minutes following infusion. All animals received an ICV infusion of either PBS or PPA. Each bar represents group mean data for the two groups. Error bars represent +SEM. ** $p < 0.01$, PPA infused animals significantly less than PBS treated animals.

Time Spent in Centre Across 2 Sessions

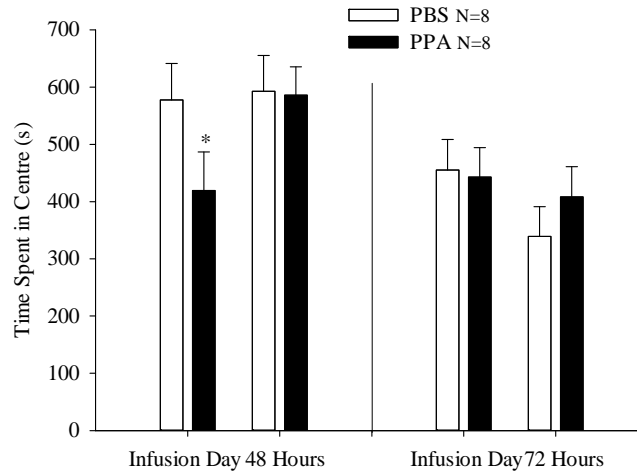
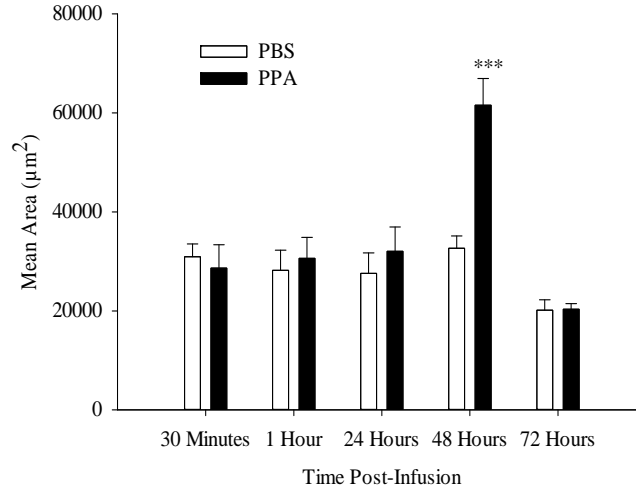


Figure 2.6: Return to baseline of thigmotaxis within 48 and 72 hours measured in animals infused with PBS or PPA. Time spent in the centre zone (s) where Infusion Day represents group means for 48 hour animals (Panel #1) and 72 hour animals (Panel #2) (N = 8 for each treatment group) on day of ICV infusion. There were no significant differences between Infusion Day PPA or PBS treated 72 hours animals and these animals did not present with increased duration after recovery. * $p < 0.05$, PPA treated 48 hour animals on Infusion Day significantly less than PBS on Infusion Day and PPA/PBS 48 hours later.

A- GFAP Immunopositive Staining in CA3/Dentate Gyrus



B- GFAP Immunopositive Staining in CA1/CA2

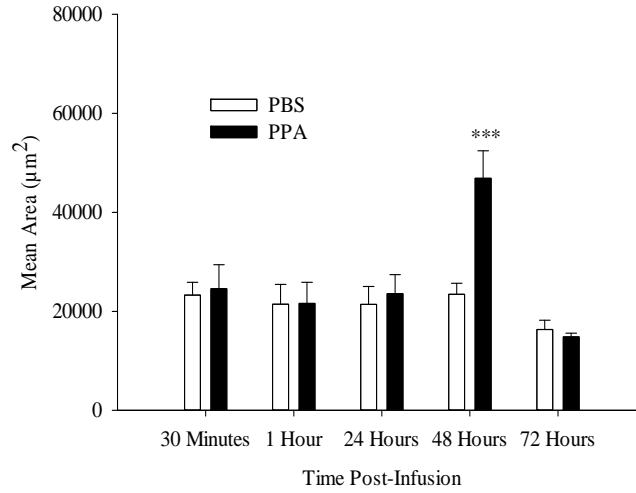


Figure 2.7: Timeline of glial cell activation (µm²) across 5 perfusion time points.

C- GFAP Immunopositive Staining in the White Matter

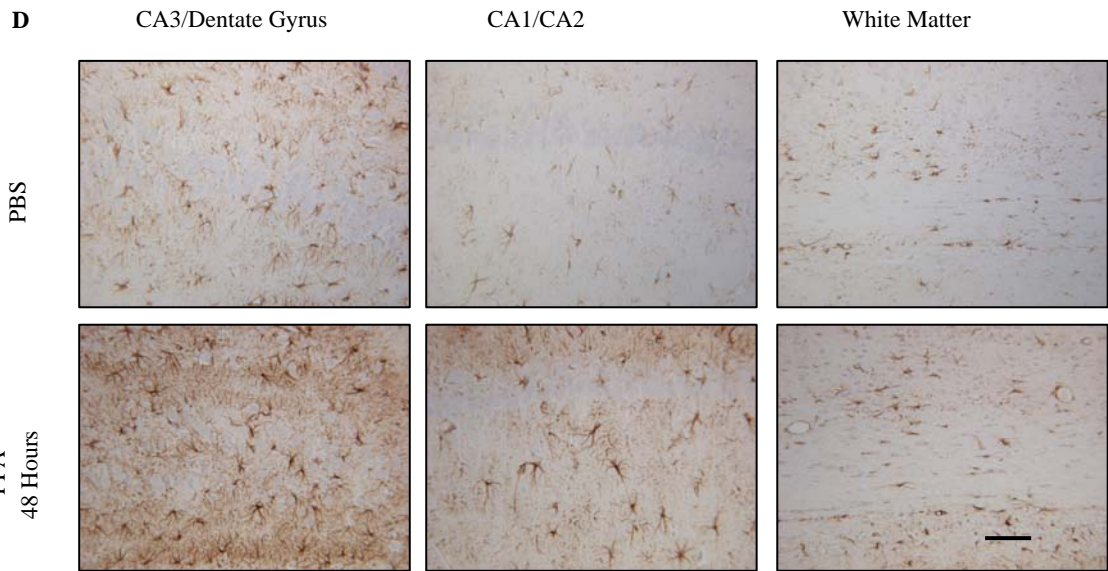
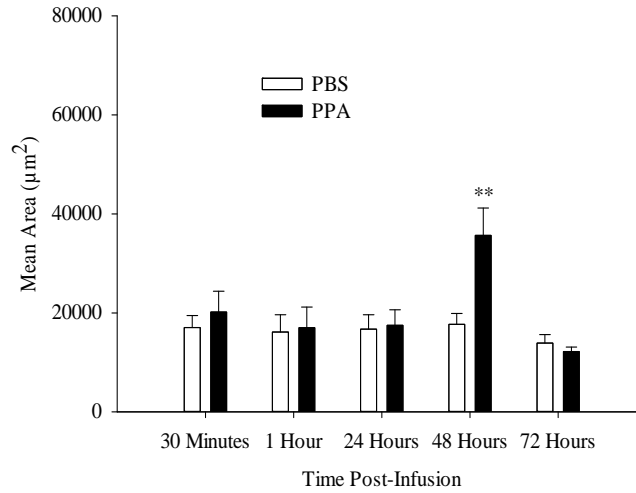
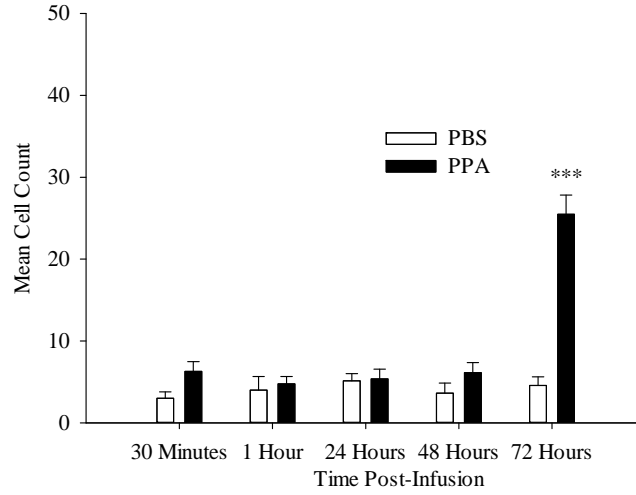


Figure 2.7 cont.: Timeline of glial cell activation (μm^2) across 5 perfusion time points and two drug groups, revealed that the greatest mean area occurred at 48 hours in the animals infused ICV with PPA ($N = 8$ except 30min/PPA, 30min/PBS, and 24hrs/PPA where $N = 7$). This significant interaction was visible in all evaluated brain areas including CA3/dentate gyrus (A), CA1-CA2 of the hippocampus (B) and the white matter of the external capsule (C). Each bar represents group mean data for the 10 treatment groups. Error bars dictate $+SEM$. Qualitative analysis (D) provided support for using the “area stained” function such that increased GFAP resulted in greater branching and hypertrophic cell bodies. *** $p < 0.001$, ** $p < 0.01$ 48 hours after PPA infusion significantly larger compared to all other treatment groups. (Scale bar is equal to $100 \mu\text{m}$)

2.3.2.2 Activated Microglia (CD68)

Similar to the astrocytic response, there was a drug by perfusion time interaction in the dentate gyrus ($F(4,68) = 23.47, p < 0.001$) and the CA1-CA2 ($F(4,68) = 12.48, p < 0.001$). The astrocytic response was not displayed in the white matter however, which indicated no significant differences in drug ($F(1,68) = 0.05, ns$), perfusion time ($F(4,68) = 0.64, ns$) or their interaction ($F(4,68) = 0.85, ns$). Likewise, MANOVA and Tukey's posthoc revealed that the mean microglial cell count was greatest at 72 hours after infusion of PPA (Dentate gyrus: $F(9,68) = 28.91, p < 0.001$; CA1-CA2: $F(9,68) = 15.29, p < 0.001$) in contrast to the 48 hour activation of astrocytes. These results are shown in Figure 2.8A-2.8C. Additionally, qualitative evidence supported the proposal that low-grade activated microglia represent cell proliferation and not hypertrophic processes (Fig. 2.8D).

A- CD68 Immunopositive Staining in CA3/Dentate Gyrus



B- CD68 Immunopositive Staining in CA1/CA2

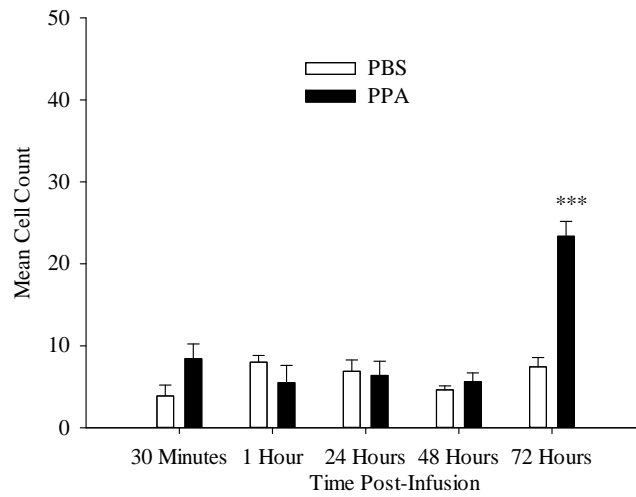


Figure 2.8: Timeline of CD68 staining across 5 different perfusion time points

C- CD68 Immunopositive Staining in the White Matter

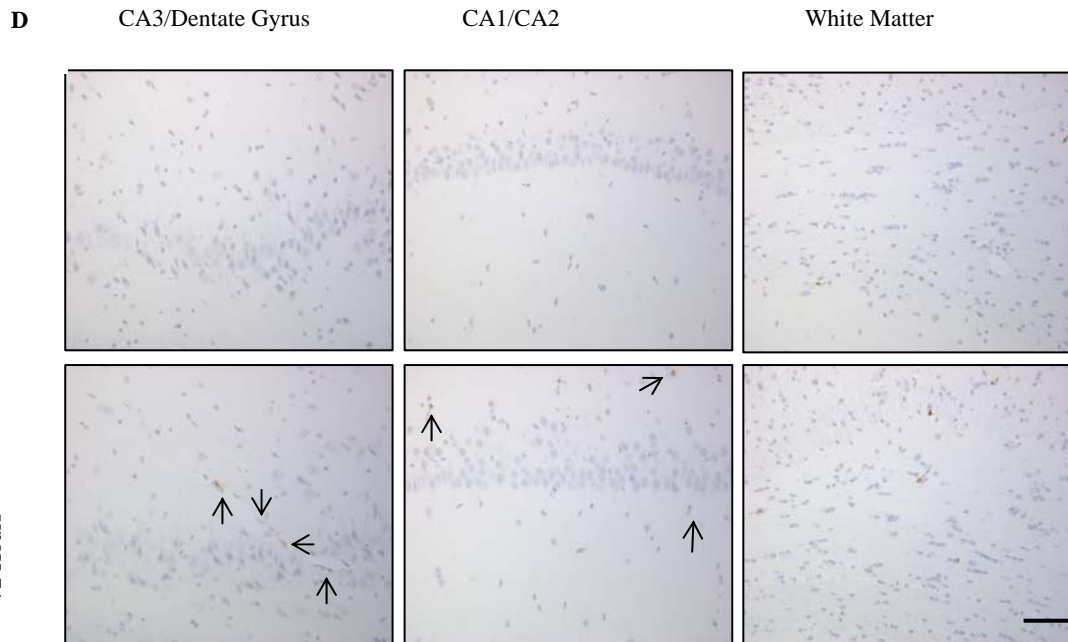
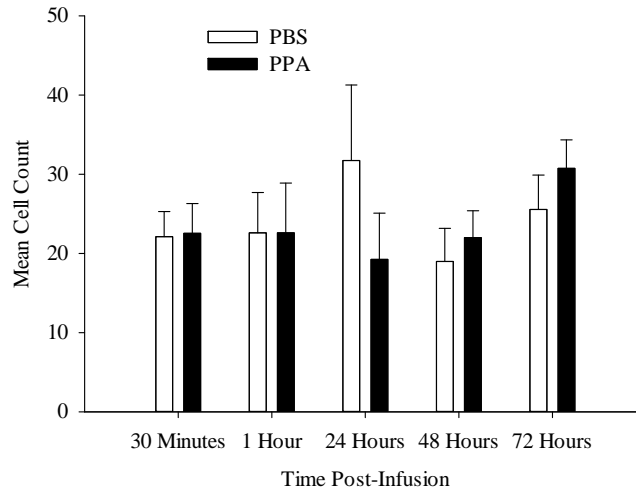


Figure 2.8 cont.: Timeline of CD68 staining across 5 different perfusion time points and two drug groups, indicated that the greatest mean cell count occurred at 72 hours in the animals infused ICV with PPA (N = 8 except 30min/PPA and 72hrs/PBS where N = 7). This result was significant in the dentate gyrus (A) and CA1-CA2 (B), whereas the white matter (C) produced no significant differences across treatment groups. Each bar indicates group mean data across drug and perfusion time point. Error bars represent +SEM. Images (D) supported small cell counts of active microglia in the dentate gyrus and CA1-CA2 and arrows point to activated microglia. *** $p < 0.001$, 72 hours after PPA infusion significantly greater than all other treatment groups. (Scale bar is 100 μ m)

2.4 Discussion

The present study found that animals infused once with PPA displayed increases in locomotor activity, anxiety-like behaviours: thigmotaxis, astrogliosis and microglial activation. By characterizing the temporal relationships between behavioural and central changes, it was shown that PPA produced rapid changes in locomotor behaviour but more delayed changes in markers of neuroinflammation. More specifically hyperactivity and thigmotaxis were altered within minutes, while astrocytes and microglia were modified across 48 to 72 hours, respectively.

2.4.1 Behavioural Parameters

Male rats treated with ICV PPA displayed significant increases in total distance traveled and mean velocity in a novel open field. This finding is the first evidence of hyperactivity following a single ICV infusion of PPA and this rapid time course is consistent with the cerebral pharmacokinetics of PPA. Brusque et al. (1999) reported the half-life of systemic administration of PPA to be between 18-57 minutes. Similarly, MacFabe et al. (2007) reviewed pilot data where ICV infusions of PPA resulted in hyperactivity within 30 minutes. This finding has not been consistently reproduced in the PPA animal model however, indicating that dose, avenue of administration (IP vs ICV), number of infusions, behavioural apparatus and experimental procedure may mediate PPA-induced locomotor activity. For example Shultz et al. (2008) injected rodents ICV once a week for two weeks and found no differences in activity as compared to controls in an open field. In contrast delivering PPA through an intraperitoneal route (IP) for two weeks, resulted in hypoactivity. Additionally, MacFabe et al. (2007) showed that central PPA injections given twice a day for 7 days produced significant increases in distance traveled only until the 6th day of injection. Finally, a study by Borlongan and colleagues (1997) found that when rodents received two IP injections of 3-nitropropionic acid, a propionyl derivative, they exhibited hyperactivity but when given four IP injections exhibited hypoactivity. These previous findings have potential confounds however in that animals were tested in pairs (Shultz et al., 2008) and IP PPA injections can induce internal aversive cues (Benzaquen et al., 2010; Borlongan et al., 1997; Ossenkopp et al., 2012). In controlling for these factors, the current findings of increased locomotion are probably representative of the immediate central effects of PPA. Further clarification, however on the relationship

between route of administration, number of infusions, and time between infusions is necessary.

In order to further examine these changes in locomotor activity, animals perfused at 48 and 72 hours were placed back into the open field to measure total distance traveled and average velocity on drug-free days. There was a significant effect such that the PPA treated animals demonstrated increased distance traveled and average velocity within 30 minutes of ICV infusion, but not when reassessed 48 and 72 hours later, when the open field was familiar. Human disorders implicating PPA such as Propionic acidemia and ASD, do display fluctuations in motor activity with periods of hyperactivity and lethargy (Feliz, Witt, & Harris, 2003; Rossignol & Frye, 2012), further validating the current findings. Regardless of the underlying rationale for the return to baseline following drug-free days, this result does suggest that PPA, whether directly or indirectly, induces rapid changes that mediate locomotor activity.

Thigmotaxis is based on the premise that in nature, rodents show an increased tendency to stay in close proximity to objects and the perimeter of an environment (Treit & Fundytus, 1989). By measuring the duration spent in the periphery or centre of a novel open field, anxiety-like behaviours can be evaluated. The PPA injected animals spent significantly less time in the centre of the novel open field relative to PBS control animals following ICV infusion. Similar studies evaluating ASD animal models reported increased anxiety with PPA systemic injections (Benzaquen et al., 2010) and pre-natal valproate exposure (Markram, Rinaldi, La, Sandi, & Markram, 2008), indicating that compounds structurally similar to PPA may also mediate levels of anxiety. Coinciding with the current finding, there is an abundance of literature implicating bacterial metabolites and microbes as causal in altering animal behaviour and modifying mood (Bested, Logan, & Selhub, 2013). Specifically, elevated levels of D-lactic acid produced from microbial species, are related to increased aggression and anxiety in rodents (Hanstock, Clayton, Li, & Mallet, 2004) and LPS from gram negative bacteria can produce cytokines that impact anxiety and depression (Reichenberg et al., 2001).

Similar to locomotor activity, there were no evident changes in thigmotaxis behaviour following reassessment. It is unclear of why both drug-free days would not result in a

return to baseline however there seems to be variability in the level of anxiety immediately following infusion. It is possible that receiving an infusion of PBS or PPA is anxiety-provoking in itself, which would explain the discrepancy between PBS treated animals analyzed at 48 hours as compared to those analyzed at 72 hours. Similarly, it is possible that group sizes were insufficient following 48 and 72 hours to display an effect. Generally, PPA induces anxiety-like behaviours immediately following infusion that are not observed on drug-free days.

The current study did find significant alterations of astrocyte and microglial markers in regions that control learning and memory. This indicates that PPA could affect long-term outcomes but hyperactivity may not necessarily be one of these behaviours associated with permanent changes. In an ASD animal model using LPS to mimic bacterial immune activation, it was found that different behaviours have different windows of susceptibility (Malkova, Yu, Hsiao, Moore, & Patterson, 2012), supporting the hypothesis that bacterial metabolites may differentially affect behaviours. Similarly, Depino, Lucchina, & Pitossi, (2011) overexpressed Transforming Growth Factor-B1 (TGF-B1), a cytokine previously shown to be increased in ASD postmortem brain tissue, in the rodent hippocampus and found increased frequency of repetitive behaviours in adulthood but reduced frequency in adolescent rats.

2.4.2 Immunohistochemistry

In the current study there was significant evidence of innate inflammation in the hippocampus (CA1-CA2), dentate gyrus (CA3-DG) and partial changes in the white matter, with the presence of these markers being temporally distinct. The idea that astrocytes and microglia become activated along different time frames is not a novel finding, however the appearance of reactive astrocytes prior to microglial proliferation is an uncommon result. For example, it is well supported that both astrocytes and microglia cells are necessary components of the host immune response, however most researchers evaluating the temporal relationships between immunomolecules have found that microglial proliferation precedes astrogliosis (Chen, Pickard, & Harris, 2003). Most of these findings have been in a stab wound model (Norton, 1999), however, so caution must be taken in drawing comparisons to the current study.

Quantitatively, the PPA-infused rodents exhibited increased expression of GFAP at 48 hours, with qualitatively different astrocytic processes and hypertrophic somas as compared to PBS rats (see Chapter 3 Fig.3.9). This embodies the definition of mild to moderate reactive astrogliosis provided by Sofroniew and Vinters (2010), where the predominant feature was described as being hypertrophy without proliferation of glial cells. Although not directly evaluated through cell count measures, it is assumed, based on qualitative evidence that proliferation has not occurred in the present experiment. Additionally, within this category of mild to moderate astrogliosis, the reaction is normally focalized to the area of impact and usually associated with viral infections and systemic bacterial infections (Sofroniew & Vinters, 2010).

What was unanticipated however, was the time frame in which one infusion was able to produce this response. A thorough literature search only found one experiment in which early activation of astrocytes was reported. Using MPTP, a dopaminergic neurotoxin, these researchers found that astrogliosis was present within one day post-exposure (O'Callaghan, Jensen, & Miller, 1995). It appears though that the dose of MPTP was intrinsically toxic in that there were significant neuronal losses causing death in a subset of animals. In contrast, the rodents in the current experiment were still fully functional as determined by their abilities to display locomotor activity. It is thus noteworthy that there was evidence of an innate inflammatory response in otherwise seemingly healthy animals.

Interestingly, GFAP levels returned to baseline values within 24 hours of attaining this peak in immunoreactivity. O'Callaghan, Jensen and Miller (1995), who were fundamental to the introduction of GFAP as an immunomarker, advise caution when interpreting the loss of immunoreactivity as being clinically relevant. It was noted that this loss could be simply a downregulation in gene expression. However it was also acknowledged that this is more likely to occur with repeated administrations of a toxin. Similarly, Chen and Swanson (2003) noted that even though astrocytes are susceptible to mild acidosis, which could be a direct result of PPA administration, irreparable damage has only been reported in chronic periods of low pH. Likewise because this reaction is categorized as mild to moderate, there are rarely permanent changes to tissue architecture in the immediate vicinity of the insult (Sofroniew, 2009). In light of this, and acknowledging that only a

single infusion of PPA was given, it can be inferred that the effects on reactive astrocytes are real but most likely transient. This does not translate into a loss of innate inflammation however, as microglia become activated within the same time period that astrogliosis dissipated.

Quantitatively, the microglia exhibited significantly increased cell counts 72 hours after PPA infusion as compared to any other time point or drug group. This effect was observed in the dentate gyrus and hippocampus whereas microglial activation did not reach statistical significance in the white matter of the external capsule, confirming the localization of a response to the site of infusion. A potential explanation for the non-significant trend of microglial activation in the external capsule is that PPA differentially targets grey and white matter. Or it could be possible that the location of the implanted cannula allows the grey matter to be influenced first. Qualitatively, the microglia present in the PPA rodent brain have a small, defined soma with long processes. This corresponds to the early stages of microglial activation and recall that a hypertrophic soma is indicative of late stages (Dihne et al., 2001). To further evaluate the stage of microglial activation, the examination of nuclear transcription factor NF- κ B may be useful as it has been shown to be overexpressed during early microglia activation (Kaltschmidt, Kaltschmidt, & Baeuerle, 1993). Analysis of interleukin 1 (IL-1) and interleukin-6 (IL-6) may also provide insight into stage as these cytokines are readily produced by microglia and are subsequently potent activators of NF- κ (Gehrmann et al., 1995). If IL-1 and IL-6 are abundantly found, it would confirm the hypothesis that PPA-induced microglia proliferation represents an early stage of activation. Concomitantly, IL-6 has been found to be significantly increased in rodents that were orally administered PPA for 3 days (El-Ansary, Ben, & Kotb, 2012).

Although there has been an absence of published studies analyzing the temporal relationship between ICV infused PPA and microglia, some researchers have characterized microglia activation in response to the central infusion of a bacterial cell wall component, lipopolysaccharide (LPS; Montero-Menei, 1996). LPS has been used to study inflammation in ASD and therefore may contribute to the understanding of PPA's role in inflammation. In general, the microglial response to injury and/or foreign

substances has been characterized as rapid although there are disagreements in what constitutes rapid changes (Davalos et al., 2005). Montero-Menei and colleagues (1996) published a study that compared the temporal activation of microglia in a traumatic injury animal model (stab lesion) and a toxin injury model (LPS infusion). It had been previously shown that one central infusion of LPS, induced inflammation with significant activation of brain macrophages at 24 hours, however the researchers were unsure of the inflammatory process before or after this time-point (Montero-Menei et al., 1994). Therefore Montero-Menei et al. (1996) focused on the microglia activation at 5, 15 and 24 hours following a single LPS infusion, as well as 3, 7 and 15 days later. It was found that with LPS infusion, inhibition of monocyte recruitment occurred at 5 hours, proceeded by substantial enhancement in microglial proliferation within 15 to 24 hours. From three days onward, the results suggested that the monocytes had transformed into macrophages, and these persisted for several weeks. While the LPS infusion produced changes by 24 hours, the PPA-induced microglial activation was not apparent until 72 hours. Because LPS was infused directly into the hippocampus with a dose twice that of PPA, this is not surprising. Taken together, LPS infused centrally appears to activate microglia along a similar timeline as a PPA infusion with a deficiency of activation at early time-points, and enhanced proliferation later on. The findings from Montero-Menei do suggest that the PPA timeline should include additional time points between 1 hour and 24 hours.

Similarly, it is important to note that while statistically significant, on average only 20 microglia were activated in PPA brain regions evaluated, calling into question the clinical relevance. In comparison, CD68 immunopositive staining where 5 central PPA injections were administered (Shultz et al., 2009) revealed average cell counts to be approximately 300. It is possible that activated microglia continue to proliferate after 72 hours eventually resulting in a greater cell count number. This hypothesis is supported by researchers who evaluated the microglial response in a stab wound model (Norton, 1999). It was reported that microglia initially appeared at 3 days post lesion, but did not obtain maximal values until the 4th day. Similarly the reaction was still evident 7 days following injury. Likewise the findings from Montero-Menei (1996) show that not only does activation persist but the monocytes likely transform into macrophages. It is thus necessary to extend the

timeline of the current study beyond 72 hours in order to further understand the clinical relevance of activated microglia.

2.4.3 Temporal Relationship & Potential Mechanisms

While the particular roles that astrocytes and microglia play during cerebral injury are now being elucidated, there has been an absence of literature characterizing the temporal relationship between these two types of glial cells. In particular, most researchers have focused on establishing the appearance of these cells in a physical injury, such as axotomized neurons (Chen et al., 2003; Ju, Holland, & Tatton, 1994), which is not directly relevant to the present findings. In the current study of an acute infusion, astrocytes appear approximately 24 hours before microglia. The notion that astrocytes preferentially activate microglia has been suggested by Ovanseov and colleagues (2008) in their research on the Borna Disease Virus (BDV). These researchers had previously observed that *in vitro* infection of BDV required both astrocytes and microglia in order for the associated neuropathology to develop, however the mechanisms involved were unclear (Ovanesov et al., 2006). In order to further understand the role of glial cells in infection, it was demonstrated that the co-culture of BDV-infected neurons and microglia did not result in activation until astrocytes were included in the culture. The researchers thus inferred that astrocytic infection could be an underlying mechanism of aberrant neuropathology. It was suggested that because astrocytes *in vivo* do not become directly infected by BDV, a factor produced by infected neurons activated the astrocytes and consequently the microglia. Interestingly, this can occur in the absence of overt neuronal toxicity (Ovanesov et al., 2008). Because BDV has been implicated in a number of neuropsychiatric disorders, ASD included (Lancaster, Dietz, Moran, & Pletnikov, 2007), the timeframe of appearance of innate immune markers may be directly pertinent to the current study. As such, the PPA animal model has provided substantial evidence for innate inflammation in the absence of neuronal injury (MacFabe et al., 2007; 2008; 2011).

It remains to be seen whether PPA can alter neuronal status with a single treatment, however it has been previously reported that PPA interferes with the sodium-potassium pump (Wyse et al., 1998), intracellular calcium levels (Nakao, Fujii, & Niederman, 1992) and NMDA receptor activity (de Mattos-Dutra et al., 2000), potentially causing aberrant

neural transmission. PPA has been shown to be capable of altering neurotransmitter systems such as dopamine, serotonin, GABA and glutamate (El-Ansary et al., 2012) and interestingly, astrocytes are particularly sensitive to these substrates (Gehrmann et al., 1996). A possible mechanism may be that PPA directly affects neural transmission, thereby altering produced substrates (e.g., glutamate) that are taken up by astrocytes. Activated astrocytes then change the microenvironment normally controlled by the microglia, which causes activation and proliferation. Interestingly Davalos et al. (2005) used time-lapse two-photon imaging and discovered that ATP in the extracellular space is essential for microglial proliferation and recruitment to the site of injury; both indicators of microglial activation. It was suggested that ATP could be released directly from astrocytes, implicating a potential role in microglia activation.

More investigation is needed however as a study conducted by Dihné et al. (2001) revealed that excitotoxicity primarily activates microglia instead of astrocytes. They injected quinolinic acid (QA) directly into the striatum and assessed the activation time points of microglia, using IB4 immunomarker, and astrocytes, using GFAP immunomarker. Being an NMDA-agonist, QA mimics the actions of glutamate, and thus could be indicative of the temporal relationship of glutamate toxicity. Researchers found that microglial proliferation occurred several days before astrogliosis (Dihné et al., 2001), although comparisons may be difficult based on the fact that IB4 does not distinguish between resident and activated microglia.

There are a number of obstacles to overcome when evaluating these two immune markers, with the most obvious being whether or not they are compensatory mechanisms in developmental disorders, causative factors or simply correlational. Ascertaining this is beyond the scope of this study's design however there is some indication that innate inflammation leads to permanent brain and/or behavioural deficits and thus may be primarily causative and neurotoxic. For example, during traumatic injury astrogliosis is marked by hyperplasia and scar formation consequently impeding axonal growth and differentiation (Chen & Swanson, 2003). Likewise astrogliosis tends to precede neuronal cell death (Kirchhoff et al., 2001), implicating its potential role in neurodegenerative diseases. Similarly the degree of activated microglia has been shown to correlate with

seizure activity in a graded fashion and to underlie learning and memory deficits (Kwon et al., 2013). Considering the early appearance of inflammatory markers such as CD68 and GFAP in the current study it is plausible that the long-term cognitive impairments seen in PPA treated animals exposed to a more chronic regime (MacFabe et al., 2008; 2011; Shultz et al., 2008; 2009) could be a result of abnormal glial cell activation in the hippocampus. In contrast, based on the results of this experiment, motor behaviours such as thigmotaxis and hyperactivity appear not to be affected by inflammation, although the role of subclinical levels of microglia and astrocytes cannot be excluded as an underlying mechanism

2.4.3.1 Fast-Acting Mechanisms

The discovery that the activation of immunomarkers did not occur until days after hyperactivity points to a short-term mechanism of PPA-induced locomotor activity, that is likely irrespective of overt astrocytic and microglial activation. There are a number of possible fast-acting mechanisms, and these can be grouped into two main categories: 1) early immune related factors or 2) neurotransmitter and neuroendocrine system activation.

Although astrocytic and microglial activation are unlikely to account for immediate behavioural changes, subclinical levels of immunological activation may contribute. For example, Lyte et al. (1998) discovered that when mice were orally challenged with bacteria, there was no overt immune activation, however anxiety-like behaviours were still significantly affected. Specifically, rodents presenting with a subclinical bacterial infection spent decreased time in the open arms of an elevated plus maze, and decreased time grooming as compared to control animals. Although it had been previously proposed that cytokine activation resulted in immediate behavioural modifications (Maier & Watkins, 1998), these researchers provided evidence for the contrary, proposing that IL-6 effects are more long-term (Lyte, Varcoe, & Bailey, 1998). Instead they speculated that the infection could directly influence neural pathways through neurotransmitter synthesis and release.

PPA's capacity to quickly induce intracellular acidification in glial cells and neurons (Karuri et al., 1993) may provide a feasible explanation for the association between

immune, neural and behavioural systems. Pitts & McClure (1967) presented data supporting this where human subjects given an intravenous infusion of lactic acid exhibited signs of a panic disorder including heightened anxiety. They suggested that systemic alterations in acid-base concentrations were an important factor. More recently this has been shown in rodents exposed to a diet high in fermentable carbohydrates (Hanstock et al., 2004). The fermentation process produces propionic and lactic acid (Arora, Sharma, & Frost, 2011) and it was found that the concentration of these acidic compounds correlated with increased anxiety as measured in a light/dark box (Hanstock et al., 2004). Even though these are systemic effects, PPA can cross the gut-blood and blood-brain barrier to alter CNS activity. Therefore, the acidic properties of PPA may mediate behaviour possibly through the modification of neurotransmitters and neuroendocrine molecules such as dopamine, glutamate, GABA, serotonin, epinephrine and norepinephrine. Results from El-Ansary et al (2012) found decreased levels of all of these compounds when PPA was administered orally.

2.4.4 Relevance to Neuropsychiatric Disorders

Even though one exposure to any chemical compound is unlikely to be causative in a neurodevelopment disorder such as ASD, the fact that PPA can produce 1) hyperactivity, 2) anxiety-like behaviour and 3) neuroinflammation upon a single ICV injection suggests that it is a potent mediator of a variety of neural pathways. This particular design could mimic the behavioural fluctuations seen in patients (Cubala-Kucharska, 2010), especially bearing in mind the return to baseline following drug metabolism. Correspondingly, anecdotal reports have indicated that when ASD children consume the complex carbohydrates that fuel PPA-metabolizing bacteria, their motor behaviours worsen (Coury et al., 2012; Horvath, Papadimitriou, Rabsztyn, Drachenberg, & Tildon, 1999). Specifically, aggression, hyperactivity, anxiety, speech impairments, posturing and toe walking seem to be abated when these compounds are removed from their diets (Jyonouchi et al., 2002).

The idea that ASD is a disorder of glial function has been extensively promoted. Accordingly, researchers have reported two phases of aberrant brain growth: a reduced head size at birth followed by a significant increase between 1 and 2 months, and then

again at 6 months (Courchesne, 2004). Similarly Carper, Moses, Tigue, & Courchesne (2002) noted age-related effects such that children with ASD had pronounced hyperplasia around 2-3 years of age followed by hypoplasia in grey and white matter. Overall these imaging studies were unable to provide an adequate explanation for the initial overgrowth and protracted development, but more recently it has been suggested that activated microglia and astrocytes are causal factors (Pardo, Vargas, & Zimmerman, 2005). They proposed that abnormalities in cytoarchitecture, such as increased cell density of small neurons (Wegiel et al., 2010), could be accounted for by these immune cells. Post-mortem brain tissues of 11 patients were analyzed and there was evidence of chronic inflammation with hypertrophic astrocytes and phagocytic microglia (Pardo et al., 2005; Vargas et al., 2005). Additionally, CSF cytokine profiling indicated high levels of cytokines and chemokines including IL-6, MCP-1 and MCP-3; potent proinflammatory molecules (Vargas et al., 2005). Because of astrocytes extensive role in maintaining an environment conducive to neuronal survival (Chen & Swanson, 2003), it can be inferred that damaged or reactive glial cells would negatively affect neuronal function and numerous studies have found that the cytokines and chemokines released from these markers alter neural transmission (Li et al., 2008; Wei & Lin, 2009; Zimmerman et al., 2005). Interestingly, PPA administered orally (El-Ansary et al., 2012) and ICV (Foley et al., 2008) has been shown to increase IL-6 production, implicating downstream markers of innate inflammation in the animal model. (Ashwood et al., 2011) also established that increased IL-6 could modify clinical course in ASD patients with these children exhibiting increased stereotypic movements. Therefore a likely consequence of astrocyte and microglia activation is the induction of damaging cytokines and chemokines. Support for PPA as a modifier of neuroinflammation is also found in post-mortem brain tissues of Propionic acidemia patients (Feliz et al., 2003). Neuroglia were noted in cortical and subcortical areas of postmortem tissue, signifying that elevated levels of PPA impact neuroimmunological course.

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Chapter 3

Temporal Changes of Locomotor Activity and Neuroinflammation following Spaced Intracerebroventricular Infusions of Propionic Acid in rats.

3.1 Introduction

3.1.1 Autism Spectrum Disorders (ASD)

ASDs are a group of neuropsychiatric disorders that affect social interaction, communication processes and motor behaviours (DSM-IV-R; American Psychiatric Association, 2000). These development disorders have been found to be comorbid with anxiety disorders (Lanni, Schupp, Simon, & Corbett, 2012), gastrointestinal complications (Horvath & Perman, 2002), disorders of biochemical pathways (e.g, methylation, lactic acidosis and oxidative stress; Cubala-Kucharska, 2010) and epilepsy (Canitano, 2007). Postmortem tissue has revealed a chronic inflammatory response characterized by activated microglia and astrocytes in the central nervous system (CNS) and an abundance of proinflammatory cytokines in the brain and cerebrospinal fluid (CSF; Pardo, Vargas, & Zimmerman, 2005; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005). The etiology of ASD has yet to be identified, however researchers have suggested that dietary, immune and metabolic factors are likely contributors (Frye, Melnyk, & MacFabe, 2013; Theoharides, Asadi, & Patel, 2013).

3.1.2 Associations between ASD and the GI tract

Gastrointestinal complications in ASD are common and researchers have described symptoms ranging from mild constipation, bloating and food sensitivities to severe abdominal pain and chronic diarrhea (Erickson et al., 2005; Horvath, Papadimitriou, Rabsztyl, Drachenberg, & Tildon, 1999). Histological reports have implicated lymphoid nodular hyperplasia, widespread inflammation through the colon, small bowel and stomach; and reflux esophagitis (Horvath et al., 1999; Horvath & Perman, 2002) as factors contributing to increased gut permeability. Dietary interventions such as reducing the consumption of gluten and casein have been shown to decrease permeability (Whiteley et al., 2012), emphasizing the important association between diet and the gastrointestinal system. Interestingly, bacteria and gluten are two of the most potent triggers for tight junction disassembly, likely resulting in altered barrier integrity (Tripathi et al., 2009).

3.1.2.1 ASD and the Microbiota

In light of the findings of alterations in gut permeability, understanding the relationship between the diet, GI tract and immune system has been a priority of many researchers (Buie et al., 2010; Coury et al., 2012). In particular, how the immune response is mediated by alterations in the microbiota or microbial substrates is now an emerging area of interest. As described by (Forsythe, Kunze, & Bienenstock, 2012), the microbiota has a distinct role in mediating stress responses. Rodents exposed to the gut inflammation-inducing parasitic species, *Trichuris Muris*, displayed increased anxiety-like behaviour as measured in a light-dark test and with elevated levels of tumor necrosis factor α (TNF- α) and interferon- γ (IFN- γ) (Bercik et al., 2010). These are potent proinflammatory cytokines that have also been demonstrated in ASD serum (Jyonouchi, Sun, & Le, 2001). In contrast, rodents exposed to beneficial commensal bacteria such as those in the *Lactobacillus* genus, can attenuate stress reactions by upregulating anti-inflammatory cytokines interleukin-10 (IL-10; Pessi, Sutas, Hurme, & Isolauri, 2000). It is thus plausible that gut dysbiosis, or imbalances in the composition of the microbiota (Petrof, Claud, Gloor, & Allen-Vercoe, 2013), can influence behavioural outcomes. This has been supported in ASD children where increased levels of *Clostridia* bacteria (Finegold et al., 2010; Parracho, Bingham, Gibson, & McCartney, 2005) and *Desulfovibrio* species (Finegold, 2011) but decreased levels of *Bifidobacterium* (Wang et al., 2011) correlate with anecdotal reports of symptom severity. Aberrant growth of toxin-producing bacteria is particularly damaging through cytokine activation (Prandota, 2010), which can induce both systemic and central inflammation (Parracho et al., 2005).

3.1.3 Inflammation as Common Pathway in ASD

A number of etiologies of ASD have been proposed including environmental exposures to heavy metals, and organophosphates, in utero exposure to ethanol, thalidomide and valproate, and reoccurring peri- or postnatal exposures to infections. Given the diversity of potential explanations for ASD, it is possible that these can all converge on a common pathway involving central and peripheral inflammation (Depino, 2013). Supporting this hypothesis is a study performed by Boris et al. (2007) where ASD children were administered pioglitazone, a synthetic drug that has documented anti-inflammatory properties through its ability to affect brain glial cell activation. The results indicated that

pioglitazone improved behavioural symptoms such as hyperactivity, irritability and stereotypy, and it was speculated to be through its adverse effects on proinflammatory cytokine production.

As discussed by Rodriguez and Kern (2011), neuroinflammation is characterized by the activation of astrocytes, microglia, and inducible nitric oxide synthase (iNOS). By catalyzing the production of the cell signaling molecule nitric oxide (NO), iNOS induces vasodilation, and modulates the expression of cytokines and chemokines, both of which promote the recruitment and activation of additional inflammatory markers (Monnet-Tschudi, Defaux, Braissant, Cagnon, & Zurich, 2011). In chronic inflammation, the hypertrophy of astrocytes and hyperplasia of microglia may contribute to altered neuronal migration (Laurence & Fatemi, 2005), and underlie the connectivity abnormalities seen in ASD patients. An EEG study by (Barttfeld et al., 2011) found that connectivity dysfunction was correlated with worsened symptomology in ASD children, suggesting that glial cell reactivity may also be correlated with increased behavioural abnormalities.

Once activated, microglia and astrocytes can protect the CNS by eliminating the source of damage through phagocytosis or endocytosis, respectively (Gehrmann, Matsumoto, & Kreutzberg, 1995; Ridet, Malhotra, Privat, & Gage, 1997) however this can also lead to further damage through signaling cascades and/or the recruitment of additional immunomolecules. For example, astrocytes can aggregate and develop a scar formation preventing the diffusion of damage, but in doing so can also cause prolonged deficits by isolating the tissue from repair mechanisms (Sofroniew, 2009). Astrogliosis and activated microglia can thus act as neuroprotectants or neurotoxins during the inflammatory process, however there are no known markers or substrates that differentiate these two outcomes.

3.1.4 Propionic Acid as an Underlying Component of ASD Symptomology

In light of the previous findings that ASD severity is correlated to both gastrointestinal symptoms (Adams, Johansen, Powell, Quig, & Rubin, 2011; Wang et al., 2011) and the degree of brain dysfunction (Barttfeld et al., 2011; Rodriguez & Kern, 2011), it can be inferred that a bacterial product that crosses the gut-blood/blood-brain barrier, alters levels of glutathione and nitric oxide, and activates astrocytes and microglia, would be a

putative causative factor. Propionic acid (PPA), an endogenous short chain fatty acid that is both a metabolic byproduct of enteric bacteria and an intermediary of fatty acid metabolism (Thompson et al., 1990), has been shown to impact all of these processes and is suspected to underlie a number of the behavioural and neuropathological changes shown in a subset of patients (Frye et al., 2013; MacFabe et al., 2007; MacFabe, 2012). In a rodent model, repeated infusions of PPA directly into the ventricle has been shown to mimic ASD-like behaviours such as hyperactivity, stereotypy (MacFabe et al., 2007; MacFabe et al., 2008), social dysfunction (Shultz et al., 2008), cognitive perseveration (Shultz et al., 2009) and object preference (MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011). Similarly, astrogliosis and microglial activation have been reported (MacFabe et al., 2007; 2008; Shultz et al. 2008; 2009).

It was demonstrated in Chapter 2 that a single ICV infusion of PPA induced immediate changes in locomotor activity and significant later increases in astrogliosis and microglial activation. Specifically locomotor activity and anxiety-like behaviours were increased within 30 minutes of ICV infusion and had returned to baseline following 48 and 72 hours. Additionally, astrocyte activation preceded microglial activation by 24 hours with astrogliosis achieving maximal values within 48 hours of PPA infusion. Furthermore, by the time microglial activation was apparent at 72 hours, astrogliosis had returned to baseline. The purpose of the current study was to evaluate these same parameters using a spaced, repeated infusion regime of once a week for four weeks. Additionally, because increased infusions of PPA may lead to more diffuse activation of astrocytes and microglia, an additional cortical brain region was evaluated.

3.2 Methods

3.2.1 Subjects

A total of 32 adult male Long-Evans rats were used (Charles River Laboratories, Quebec, Canada). At the time of surgery, rats weighed 250-350g and were housed in pairs in standard polypropylene cages in a colony room with a controlled ambient temperature of $21 \pm 1^{\circ}\text{C}$. Following surgical implantation of a cannula, the animals were housed individually and permitted 14 days to recover before the commencement of behavioural testing. In total the rodents spent 6 weeks independently housed. The colony room was

kept at a 12:12 hour light:dark cycle and all behavioural testing was conducted during the light cycle from 0700h to 1900h. The animals were given *ad libitum* access to food (ProLab rat chow) and water and were naïve to all experimental procedures. Housing and testing procedures were performed in accordance with the guidelines set out by the Canadian Council for Animal Care (CCAC) and were approved by the University of Western Ontario Animal Use Subcommittee (see Appendix A).

3.2.2 Surgical Implantation of Guide Cannula

All of the rats were implanted with a 23-gauge guide cannula under standard stereotaxic procedures. First rats were placed in a Plexiglas box where a 4% isoflurane and 2L/min oxygen flow allowed for the induction of anesthesia. Upon reaching an adequate depth the rats were removed from the box and placed into a stereotaxic device equipped with a nose cover that permitted gas flow during surgery. The animals were then subcutaneously injected with an analgesic (Ketoprofen, 1mL/kg). Under aseptic conditions, a cannula was implanted into the right lateral ventricle with the tip being placed according to the following coordinates in relation to Bregma: -1.4 anterior/posterior, +1.8 medial/lateral and -3.0 dorsal/ventral (Paxinos, Watson, Pennisi, & Topple, 1985). Stainless steel screws were inserted into the skull to serve as anchors for the adherence of dental acrylic to the cannula and skull. A plastic removable plug was placed in the top of the cannula in order to avoid contamination and silk sutures were used to aid in the healing process.

3.2.3 Treatment Groups

Following the recovery time, rats were randomly assigned to two equal treatment groups (N = 16 for each group) and exposed to a treatment paradigm consisting of one ICV infusion per week for four weeks. Animals received intracerebroventricular (ICV) infusions of either drug or control solution: 4µL of a 0.26M solution of physiologically-buffered Propionic Acid (PPA, pH = 7.5) or 4µL of a 0.1M solution of Phosphate Buffered Saline (PBS, pH = 7.5). The solutions were administered to the lateral ventricle via a 30-gauge injection cannula, thereby protruding 0.5mm beyond the tip of the implanted cannula. The injection cannula was connected to a Sage syringe pump by PE10 tubing and the delivery of 4µL of solution took approximately one minute to complete. The previously mentioned doses were chosen based on dose-response findings by

MacFabe et al. (2007). Each animal received a total of four pulse infusions with each pulse immediately preceding behavioural testing (see Fig. 3.1).

3.2.4 Behavioural Apparatus

Locomotor activity of individual animals was evaluated in a circular open field measuring 90cm in diameter and 40cm in height. The floor of the apparatus was covered with Beta chip bedding used to facilitate the contrast between the animal and the surrounding arena. The bedding was replenished and the chamber washed between testing days. A CD camera and darkroom lamp were located above the centre of the apparatus and the camera was connected to a computer that used *EthoVision 3.0.15 Behavioural Monitoring and Analysis System*, to quantify measures of interest. This computer program was capable of tracking the x-y coordinates of the animal and was therefore used to compute 1) total distance traveled (cm) and 2) average velocity (cm/s). Furthermore, the open field was divided into two separate zones with a centre zone equating to the interior 2/3 of the total field, and a peripheral zone equating to the exterior 1/3. This distinction was used to decipher 3) the time spent in the centre zone (s), in order to measure thigmotaxis, an index of anxiety. Additionally, the CD camera was connected to a VCR, which allowed for future analysis of behaviour.

3.2.5 Experimental Procedure (see Fig. 3.1)

In addition to being randomly assigned to a drug group, each animal was assigned to a time period dictating the interval between their fourth ICV infusion and the administration of a sub-lethal dose of sodium pentobarbital (270mg/mL IP). In this way a timeline of PPA-induced behaviour changes and neuroinflammation could be established. In light of the findings from the previous experiment described in Chapter 2, in which the 48 and 72 hour time intervals proved the most significant, these were the two time periods evaluated here. Overall there were 4 treatment groups: PPA-infused rats and PBS-treated rats perfused at 48 hours (N = 8, respectively) and PPA-infused rats and PBS-treated rats perfused 72 hours after the 4th ICV infusion (N = 8, respectively; Step 1 in Fig. 3.1).

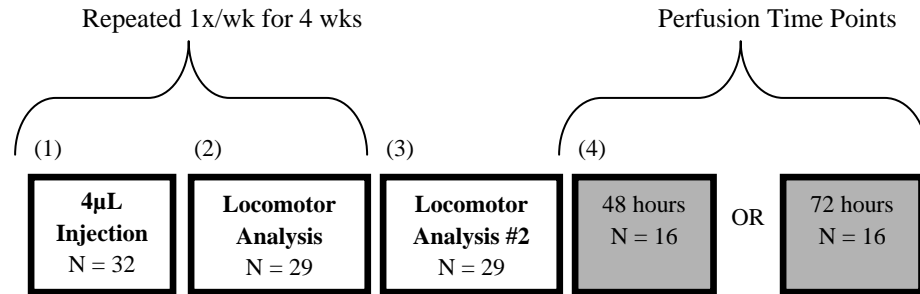


Figure 3.1: Methods Paradigm, where N = number of animals. All animals received an ICV infusion into the right lateral ventricle and were immediately placed into the open field for 20 minutes. Behavioural parameters (total distance traveled, mean velocity, and duration spent in centre zone) were computed for all of the groups. Steps 1 and 2 were repeated 4 times to decipher the timeline of a spaced treatment regime. Immediately prior to perfusion (48 or 72 hours following final infusion), behavioural parameters were reassessed and then animals were perfused based on time interval. Perfusion time points refer to the time between ICV infusion and receipt of anesthetic dose of euthanyl. Brains were then removed for further analysis.

Once the assigned drug was administered, each rat was placed in the centre of the open field apparatus for 20 minutes. Locomotor activity was monitored for this amount of time based on a pilot study by MacFabe et al. (2007) that provided evidence for the behavioural effects of PPA obtaining maximal values within the first 30 minutes post infusion. Additionally the half-life of PPA is known to be between 18 and 57 minutes (Brusque et al., 1999) further supporting this time frame. This procedure was repeated four times in order for the impact of multiple spaced PPA infusions to be determined (Step 2 in Fig. 3.1). As in the previous experiment, whether hyperactivity and thigmotaxic behaviours returned to baseline was of interest. All animals were analyzed in the open field 48 or 72 hours following final infusion and immediately preceding perfusion (Step 3 in Fig. 3.1). In total, animals received 4 ICV infusions and their behaviour was evaluated 5 times. There was a malfunction with the tracking device during week four and three of the rats' locomotor activity was not analyzed (N = 29)

3.2.6 Brain Tissue Preparation and Histological Procedures

At their assigned time interval, rats were deeply anesthetized with sodium pentobarbital (270mg/mL IP) and were transcardially perfused with 200mL of 0.1M ice cold PBS and 300mL of 4% paraformaldehyde in PBS (Step 4 in Fig. 3.1). The brains were removed, placed in paraformaldehyde for further fixation and stored at 4°C for 24 hours. Following complete fixation, the brains (N = 32) were transferred into an 18% sucrose solution for cryoprotection. All of the brains were then blocked into 5 equal 2mm sections, defatted and dehydrated through increasing concentrations of ethanol and embedded in paraffin wax. Cannula placement was ascertained visually once brains were blocked. Using a Leica microtome (model RM2125), coronal sections 4µm thick were sliced through the dorsal hippocampus and adjacent white matter (Paxinos et al., 1985), and mounted on glass slides (SurgiPath, Canada). The tissues were rehydrated and deparaffinized through exposure to xylene and decreasing concentrations of ethanol and endogenous peroxidase activity was blocked using 3% hydrogen peroxide in distilled water.

The following markers were used to determine evidence of innate inflammation: 1) Anti-glial fibrillary acidic protein (GFAP: 1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark) and 2) Anti-rat CD68 antigen (1:200, monoclonal, Serotec, Oxford,

UK). Note that antigen retrieval, in which aldehydes were removed through the immersion of tissue in 0.21% citric acid buffer (pH = 6.0) for 30 minutes, was necessary only in preparation for CD68 antibody binding. After immersion, slides were counterstained with Gill hematoxylin (EMD Chemicals, New Jersey), dipped in acid alcohol, ammonia, and rinsed with distilled water. In anticipation of antibody exposure, tissue was coated with PBS and 10% horse serum, an inert protein used to prevent antibody binding as a result of charge differential. Primary antibody (GFAP and CD68) was applied and slides were incubated for 1 hour at room temperature. Following incubation, brain tissue was rinsed with PBS, and the secondary antibody, either biotinylated anti-rabbit (GFAP 1:200, Vector Laboratories, California) or biotinylated anti-mouse (CD68: 1:200, Vector Laboratories, California) was applied for 30 minutes. Again, tissue was rinsed and then stained with avidin-biotin complex (Vectastain Elite ABC, Vector Laboratories, California) for another 30 minutes. After this final incubation, tissues were washed with PBS and 3,3-diaminobenzidine DAB chromagen (Sigma-Aldrich, Missouri) for 5 minutes. This histological procedure was completed once the tissue was dehydrated with ascending concentrations of alcohol and coverslipped.

3.2.6.1 Immunohistochemical Quantification

The imaging analysis was used according to the procedure described in previous studies (MacFabe et al., 2007; Shultz et al., 2008). It involved using a standard light microscope and taking 4 digital pictures spanning the pyramidal layer of the hippocampus from CA1 to CA2, and 4 images along CA3 to the hilus of the dentate gyrus. In addition 7 pictures were taken of the adjacent white matter for a total imaging area of 320,000 μm^2 (see Chapter 2 Fig. 2.2 for visualization). This pattern was applied to the imaging of each rat brain (N = 31 for CD68 due to incomplete tissue preparation) and a set of color recognition criteria was created to evaluate the staining. Because of the robust neuroinflammatory findings with a single infusion in the dentate gyrus, CA1-CA2 and white matter, an additional grey matter area was imaged to assess whether these results could be generalized to other brain regions. Anterior to the white matter of the external capsule, seven pictures (160,000 μm^2) were taken starting at the retrosplenial granular b cortex (RSGb). The wavelengths of red, blue and green were chosen based on their ability to capture the immunopositive staining of each individual image and were different for

each antibody to control for the variance in intensity of DAB labeling. Using ImagePro Plus software, the quantification of the staining was conducted for each brain region (CA3-dentate gyrus, CA1-CA2, white matter and cortex). To evaluate reactive astrocytes (GFAP), each image was measured for its degree of total immunopositive staining per area (μm^2), and the results of each image were summed to produce a total area in each brain region. Because microglia replicate (Gehrmann et al., 1995) while astrocytes grow larger (Ridet et al., 1997) in neuroinflammation, each image stained with CD68 yielded a total cell count. Again the results of each image were summed to produce total count per brain region.

3.2.7 Statistical analysis

To determine whether the effect of repeated PPA infusions differed from the control infusion in behaviour and neuroinflammation, several analyses were conducted. A Repeated Measures analysis of variance (ANOVA) was conducted on EthoVision locomotor data (total distance traveled, mean velocity and duration spent in centre zone) with drug treatment (2 levels: PPA and PBS) as the between-subjects factor and injection week (4 levels: Week 1, Week 2, Week 3 and Week 4) as the within-subjects factor. Similarly, a Repeated Measures design was used to evaluate the return to baseline of behavioural parameters in the 48 hour and 72 hour groups, separately. In this case, Session (2 levels: #1 and #2), with #2 equating to either 48 or 72 hours after the final infusion, was the within subjects variable whereas drug (2 levels: PBS and PPA) was the between-subjects variable. A 2 x 2 ANOVA was also conducted for mean area of GFAP and cell count of CD68 in the dentate gyrus, CA1-CA2, white matter, and cortex. Drug treatment (2 levels: PPA and PBS) and perfusion time (2 levels: 48 hours and 72 hours) were the between subjects factors.

To further understand the temporal effects of neuroinflammation, the repeated acute (4 infusions) and single acute (1 infusion: data from experiment #1) paradigms were compared. This resulted in two additional analyses being conducted. A 2 x 2 ANOVA was performed evaluating the difference between 1 infusion and 4 infusions in reactive astrocytes at 48 hours (between subject factors being drug: PPA and PBS, and number of infusions/infusion frequency: 1 vs 4), and again at 72 hours. The difference in mean

immunopositive area was analyzed across the dentate gyrus, CA1-CA2 and white matter. Likewise, a 2 x 2 ANOVA was also conducted for microglial proliferation comparing drug reactions across number of infusions. These changes were calculated at 48 hours and 72 hours separately, and in the dentate gyrus (CA3-DG), CA1-CA2 and white matter. Multivariate ANOVAs (MANOVA) and Tukey's HSD post-hoc analysis were conducted where appropriate. An alpha level of 0.05 was used as the significance criterion.

3.3 Results

3.3.1 Behaviour

3.3.1.1 Locomotor Activity

The first measure to assess activity, total distance traveled, resulted in a significant main effect of drug ($F(1,27) = 8.94, p < 0.01$) but not a significant drug x week interaction ($F(3,81) = 0.42, ns$). As shown in Figure 3.2A, either a tolerance to PPA was not produced, drug-infused rodents did not habituate to the apparatus or did not recognize the open field as familiar, as the PPA group traveled a greater overall distance than the PBS control animals. Similarly, analysis of the average velocity revealed a significant main effect of drug ($F(1,27) = 8.94, p < 0.01$) and a non-significant interaction ($F(3,27) = 0.42, ns$). Figure 3.2B shows this result indicating that PPA infused animals were significantly faster than PBS infused animals in general. Overall, the consequence of PPA infusion: hyperactivity, did not appear to abate over multiple infusions presented one week apart.

Interestingly when the rodents were re-evaluated in the open field following 48 ($N = 15$) and 72 hours ($N = 14$) of no drug, there was no significant difference between the final infusion and assigned recovery. At 48 hours post-infusion, total distance traveled resulted in a non-significant drug effect ($F(1,13) = 1.58, ns$), session effect ($F(1,13) = 1.12, ns$), and drug by session interaction ($F(1,13) = 0.02, ns$). Likewise, 72 hours post-infusion did not yield significant differences in drug ($F(1,12) = 1.65, ns$), session ($F(1,12) = 1.25, ns$), or interaction ($F(1,12) = 0.98, ns$). These findings were similar to velocity where there was no return to baseline within 48 hours (drug: $F(1,13) = 1.58, ns$, session: $F(1,13) = 1.12, ns$, interaction: $F(1,13) = 0.02, ns$) nor 72 hours (drug: $F(1,12) = 0.17, ns$, session: $F(1,12) = 1.25, ns$, interaction: $F(1,12) = 0.98, ns$). In contrast to the results in Chapter 2, there were no significant differences in locomotor activity upon reassessment (see Fig 3.3).

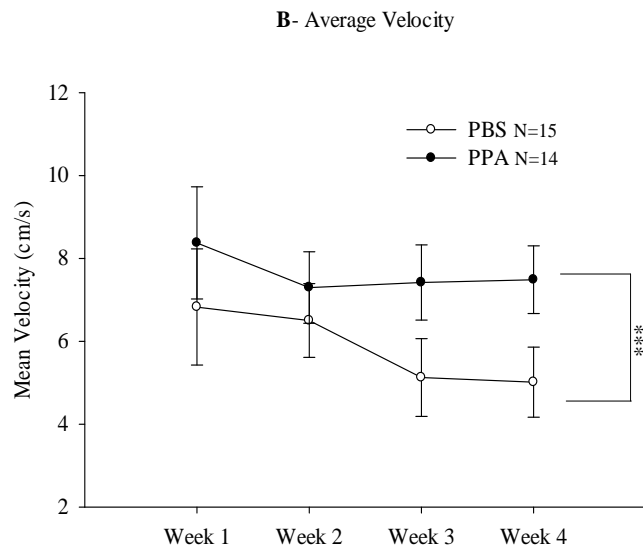
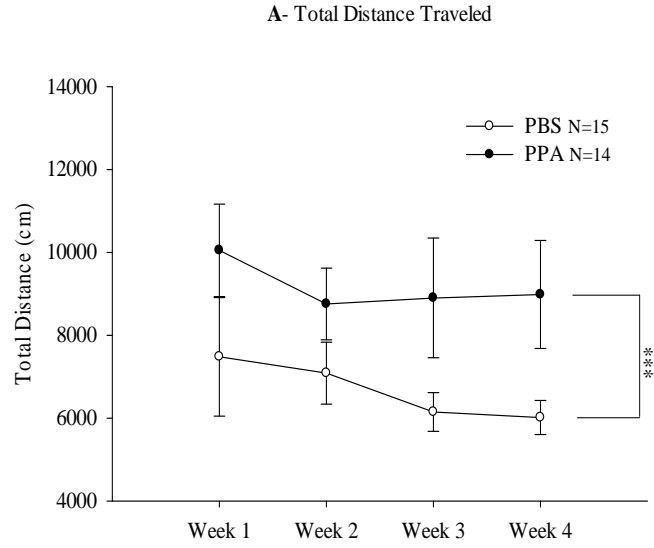


Figure 3.2: (A) total distance traveled (cm) and (B) mean velocity (cm/s) in an open field of animals infused ICV with PBS or PPA. All animals received four infusions and were situated in the open field immediately following treatment. Each symbol represents group mean data for the 20 minutes that the animals were evaluated each week. Week corresponds to number of injection such that Week 1 represents the first infusion, Week 2 the second and so on. Error bars are +SEM. ** $p < 0.01$, PPA infused animals significantly greater than PBS treated animals at weekly infusions.

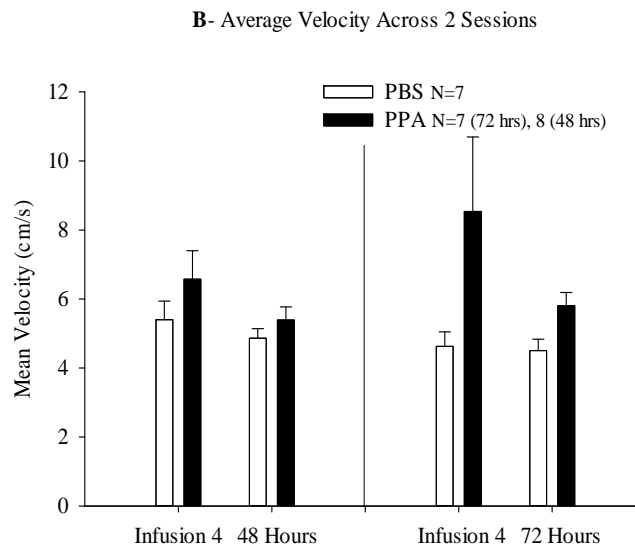
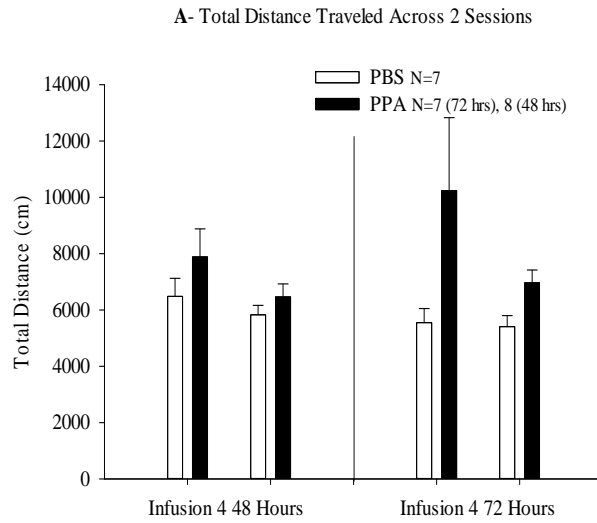


Figure 3.3: Analysis in 48 and 72 hour animals with (A) total distance traveled (cm) and (B) mean velocity (cm/s) measured in an open field across animals infused ICV with PBS (N = 7) or PPA (N = 8 for 48 hour animals and N = 7 for 72 hour animals). Infusion 4 refers to group means of 48 hour animals (Panel #1) and 72 hours animals (Panel #2) immediately following their final infusion. There were no significant differences 48 or 72 hours later.

3.3.1.2 Thigmotaxis

Thigmotaxis measures anxiety-like behaviours through evaluating the duration of time spent in the centre zone. Unlike the previous experiment of Chapter 2, there were no significant main effects of drug ($F(1,27) = 0.01, ns$) or week ($F(3,81) = 2.55, ns$) nor was there a significant drug x week interaction ($F(3,81) = 1.17, ns$). The PPA-infused animals spent approximately the same duration in the centre zone as compared to PBS-infused animals (Fig. 3.4)

Again it was important to determine behaviour after the rodents had two or more drug-free days. The repeated measures design indicated that there were no differences in PPA or PBS infused animals between their final injection day and their reassessment. These anxiety-like behaviours did not change within 48 hours (Interaction: $F(1,13) = 0.15, ns$), or 72 hours (Interaction: $F(1,13) = 1.28, ns$) post-infusion. These results are depicted in Figure 3.5.

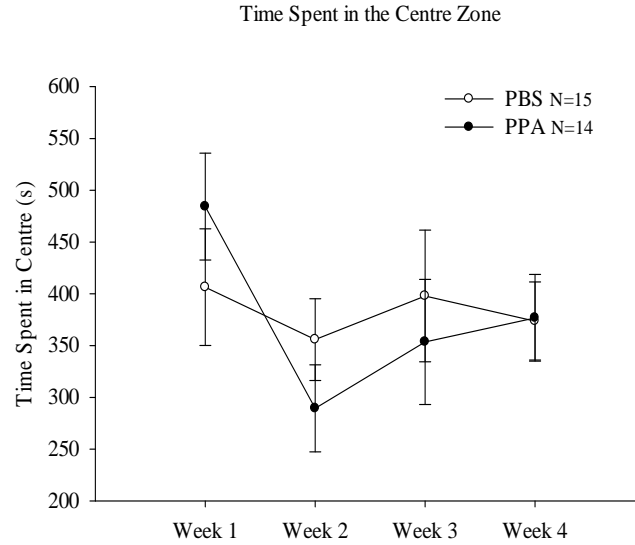


Figure 3.4: Duration spent in the centre zone (s) was evaluated in an open field for 20 minutes following each infusion. All animals received one ICV infusion of either PBS or PPA a week for 4 weeks. Each symbol represents group mean data across each infusion week. Week 1 refers to injection #1, Week 2 represents injection #2 and so forth. Error bars represent +SEM. There were no significant differences between PPA and PBS groups and their level of anxiety did not significantly fluctuate across weeks of treatment.

Time Spent in the Centre Across 2 Sessions

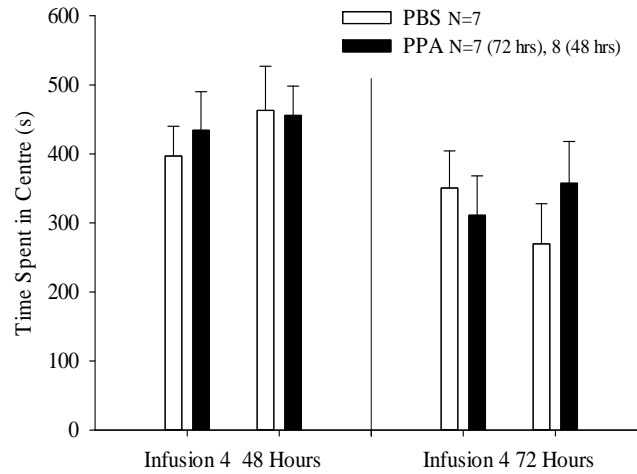


Figure 3.5: 48 and 72 hours animals injected ICV with PBS (N = 7) or PPA (N = 8 for 48 hour animals, N = 7 for 72 hour animals). Time spent in the centre zone (s) where Infusion 4 represents group means for 48 hour animals (Panel #1) and 72 hour animals (Panel #2) on their final infusion day. Error bars refer to +SEM. There were no significant differences between PPA or PBS treated animals and these animals did not present with increased duration after recovery.

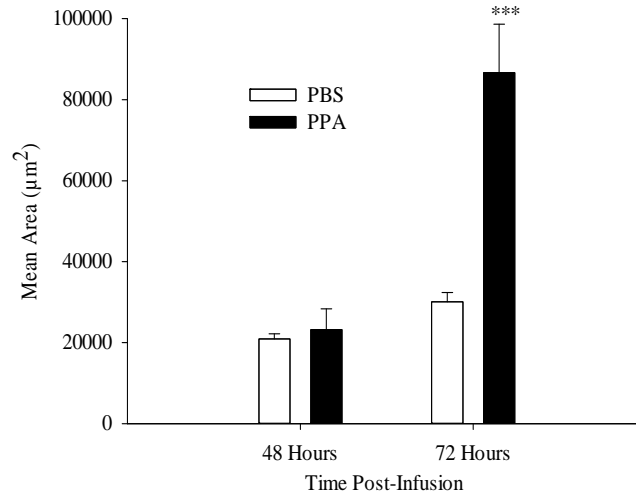
3.3.2 Neuroinflammatory Response

3.3.2.1 Astrogliosis (GFAP)

The 2 x 2 ANOVA revealed a significant drug by perfusion time interaction for the activation of astrocytes in the dentate gyrus ($F(1,28) = 16.60, p < 0.001$), CA1-CA2 ($F(1,28) = 8.57, p < 0.01$), and adjacent white matter ($F(1,28) = 7.16, p < 0.05$). Analyses also provided evidence that astrogliosis was localized as there was no main effect of drug, perfusion time or interaction in the cortex (Fig. 3.6D). Tukey's post-hoc revealed that in the dentate gyrus, CA1-CA2 and the white matter, the group of PPA treated animals perfused at 72 hours exhibited the greatest increase in immunopositive staining (Fig. 3.6A: $F(3,28) = 29.93, p < 0.001$; Fig. 3.6B: $F(3,28) = 14.10, p < 0.001$; Fig. 3.6C: $F(3,28) = 11.41, p < 0.001$) with none of the other treatment groups varying across drug or perfusion time. This is shown qualitatively in Figure 3.6E. Interestingly this maximal increase at 72 hours represented a shift compared to the 48 hour maximum found with a single infusion.

More specifically, evaluating the mean area at 48 hours between 1 and 4 infusions, a significant drug by infusion frequency interaction was demonstrated. In the dentate gyrus (Fig. 3.7A: $F(1,28) = 11.20, p < 0.01$) and the CA1-CA2 (Fig. 3.7B: $F(1,28) = 5.58, p < 0.05$), the animals receiving a single infusion of PPA exhibited the greatest mean increase in astrocytes. Post-hoc comparisons indicated that one infusion of PPA differed from all of the other treatment groups (DG: $F(3,28) = 22.08, p < 0.001$, H: $F(3,28) = 12.81, p < 0.001$). Although the white matter did not display a significant interaction there was a significant main effect of drug ($F(1,28) = 9.33, p < 0.01$) and a significant main effect of infusion frequency ($F(1,28) = 12.10, p < 0.01$) such that on average PPA had greater astrogliosis than PBS and a single infusion caused more of a reaction than four, spaced infusions (Fig. 3.7C). This relationship was reversed at 72 hours, however. Figure 3.8A shows that in the dentate gyrus, 4 infusions of PPA produced significantly more astrocyte activation than its corresponding time point and control group. This pattern was identical in CA1-CA2 ($F(1,28) = 13.72, p < 0.01$) and the white matter ($F(1,28) = 10.89, p < 0.01$), with four infusions of PPA causing significantly more astrogliosis at 72 hours than at 48 hours (Fig. 3.8B-C). Figure 3.9 represents a qualitative view of maximal astrocytic activation.

A- GFAP Immunopositive Staining in CA3/Dentate Gyrus



B- GFAP Immunopositive Staining in CA1/CA2

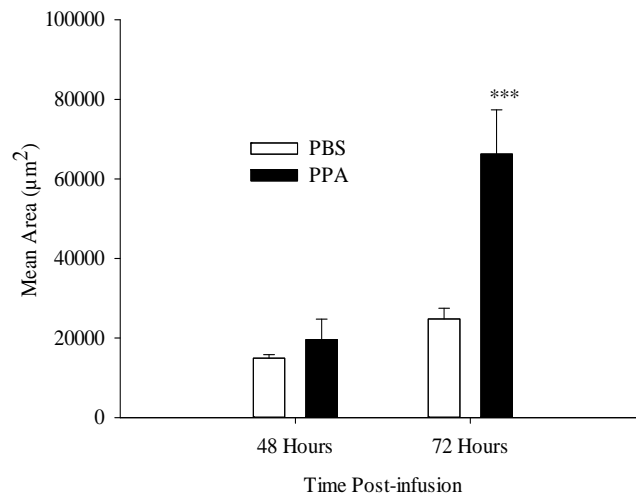
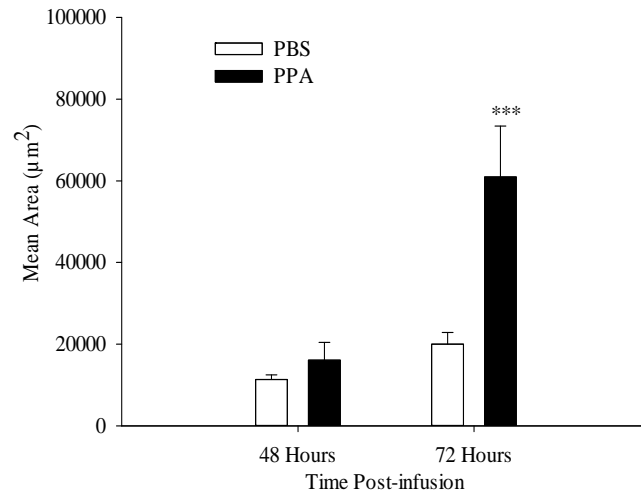


Figure 3.6: GFAP immunopositive staining (µm²)

C- GFAP Immunopositive Staining in the White Matter



D- GFAP Immunopositive Staining in the RSGb Cortex

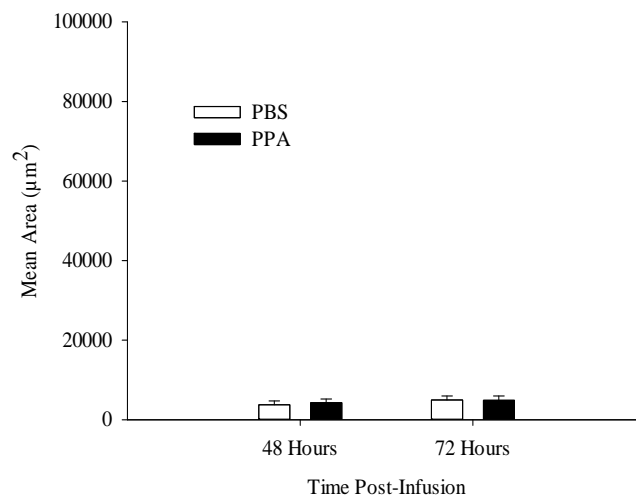


Figure 3.6 cont.: GFAP immunopositive staining (μm^2)

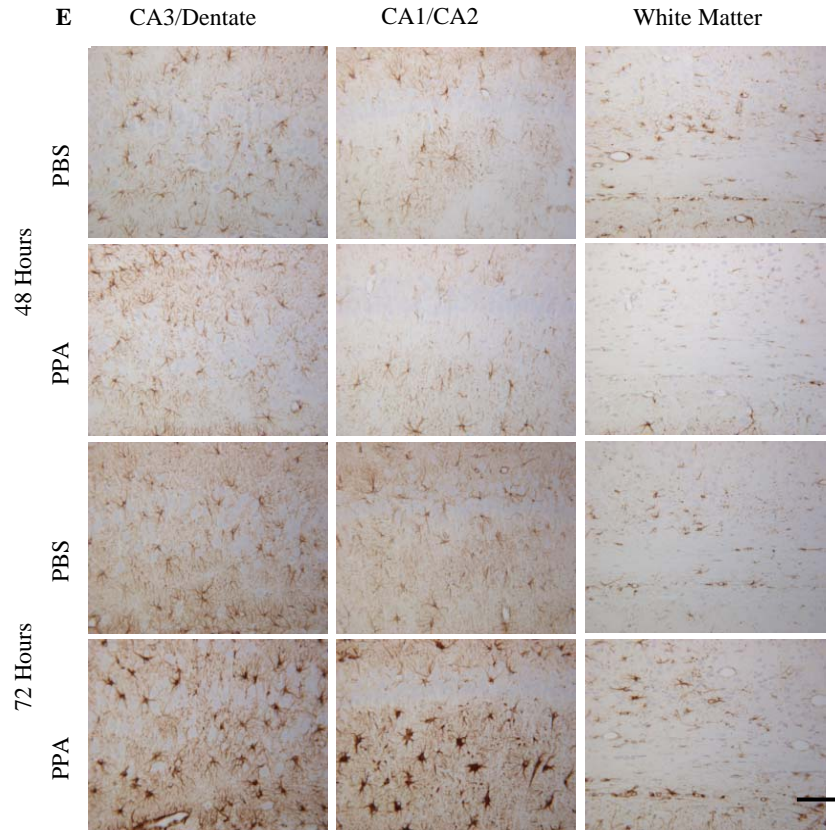
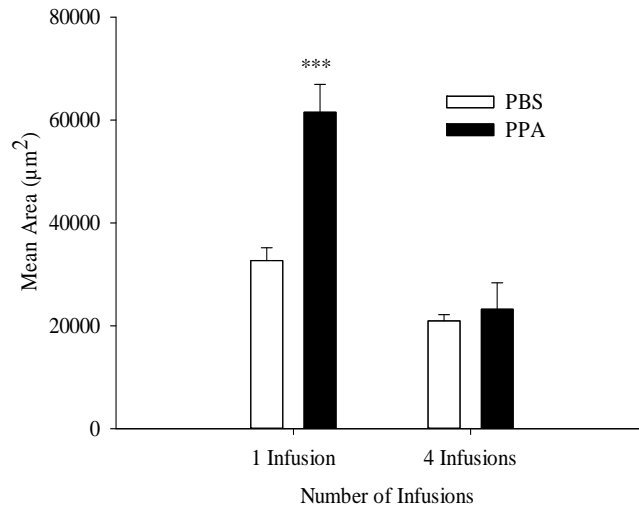


Figure 3.6 cont.: GFAP immunopositive staining (μm^2) across two perfusion time points and two drug groups in CA3/dentate gyrus (A), CA1-CA2 of the hippocampus (B), the white matter of the external capsule (C), and the cortex (D) (N = 8 for each treatment group). Pictures spanning the dentate gyrus, CA1-CA2 and whiter matter (E) demonstrated changes across time period. Each bar represents group mean data for the 4 treatment groups. Error bars display +SEM. *** $p < 0.001$, 72 hours after PPA infusion significantly larger compared to all other treatment groups. (Scale bar is equal to $100\mu\text{m}$)

A- GFAP Immunopositive Staining in CA3/Dentate Gyrus at 48 hours



B- GFAP Immunopositive Staining in CA1/CA2 at 48 hours

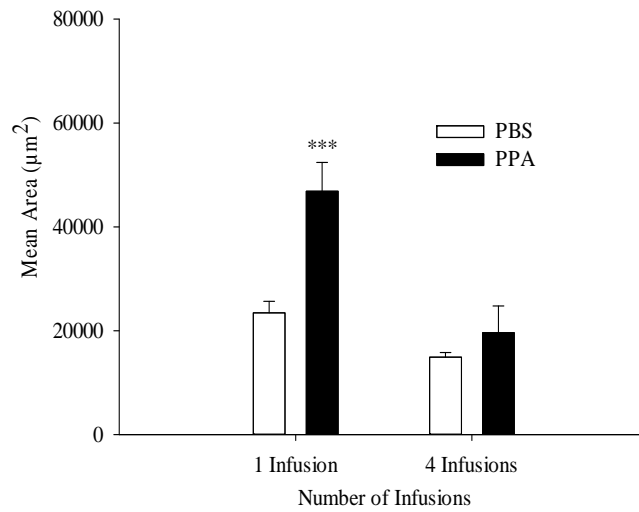


Figure 3.7: Comparisons between a single acute (1 infusion: from Chapter 2) and a repeated, spaced treatment regime (4 infusions) 48 hours following infusion

C- GFAP Immunopositive Staining in the White Matter after 48 hours

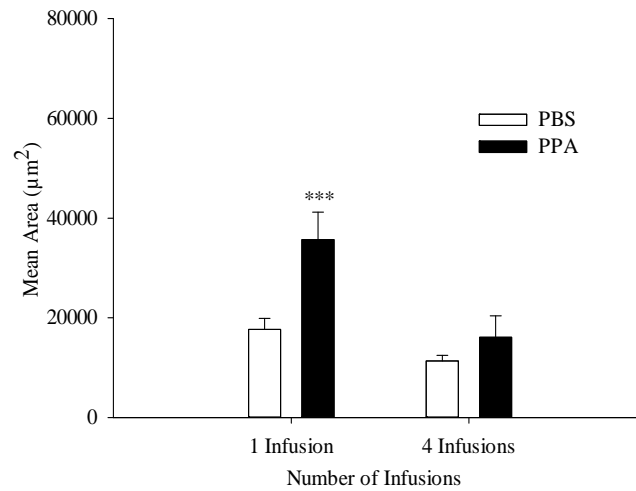
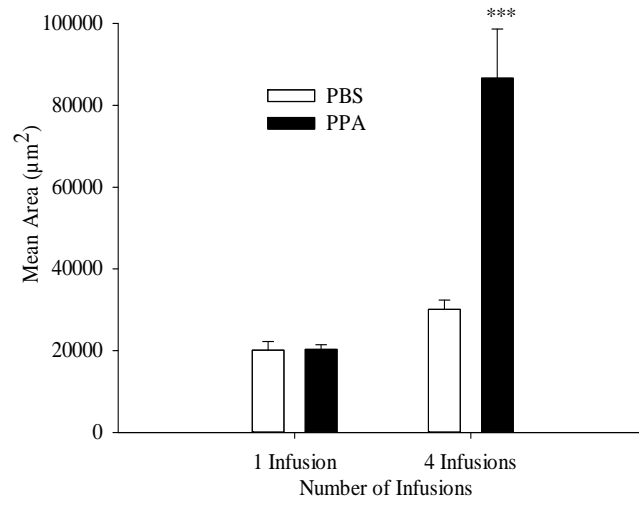


Figure 3.7 cont.: Comparisons between a single acute (1 infusion: from Chapter 2) and a repeated, spaced treatment regime (4 infusions) 48 hours following infusion of either PBS or PPA (N = 8 for all treatment groups). Measurement of GFAP immunopositive (μm^2) staining occurred in the dentate gyrus (A), CA1-CA2 (B), and the white matter (C). Each bar represents group mean data for the 4 treatment groups. Error bars display +SEM. *** $p < 0.001$, 48 hours after single infusion of PPA significantly increased compared to all other groups.

A- GFAP Immunopositive Staining in CA3/Dentate Gyrus at 72 Hours



B- GFAP Immunopositive Staining in CA1/CA2 at 72 Hours

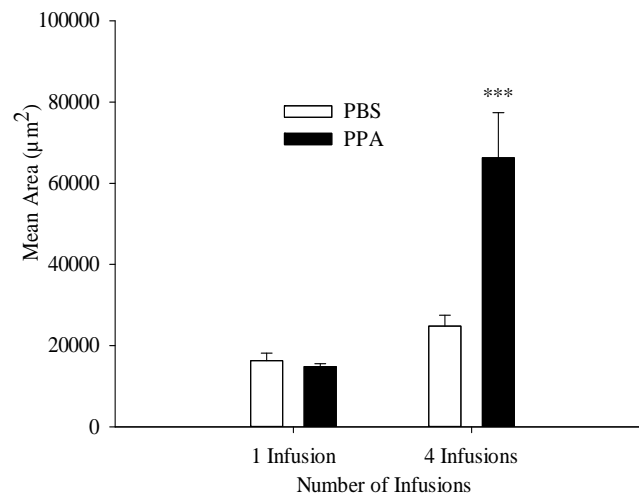


Figure 3.8: Comparisons between a single acute (1 infusion) and a repeated, spaced treatment regime (4 Infusions) 72 hours following infusion

C- GFAP Immunopositive Staining in the White Matter at 72 hours

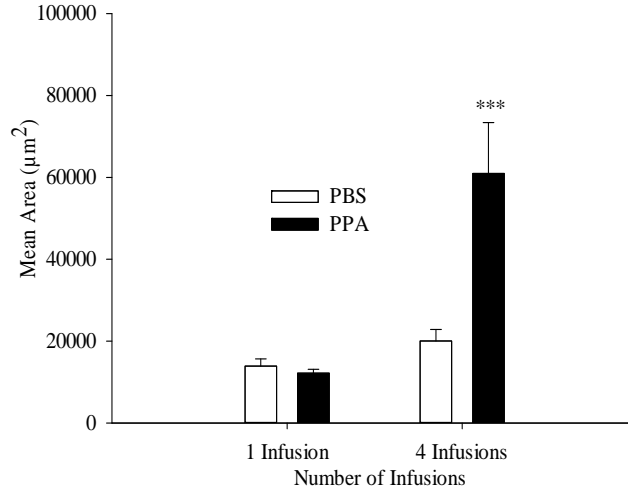


Figure 3.8 cont.: Comparisons between a single acute (1 infusion) and a repeated, spaced treatment regime (4 Infusions) 72 hours following infusion of either PBS or PPA (N = 8 for all treatment groups). GFAP immunopositive staining (μm^2) was evaluated in the dentate gyrus (A), CA1-CA2 (B) and the white matter of the external capsule (C). Each bar represents group mean data for the 4 treatment groups. Error bars display +SEM. *** $p < 0.001$, 72 hours after repeated infusions of PPA significantly increased compared to all other groups.

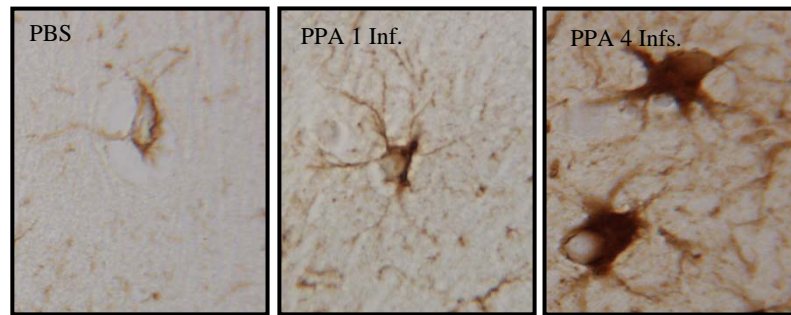


Figure 3.9: Qualitative view of GFAP following infusion of PBS or PPA. PPA 1 inf. labels image of astrocyte activation at 48 hours following single ICV infusion and PPA 4 infs. labels image of astrocyte activation at 72 hours following four spaced ICV infusions. Images were taken at an objective power of 63 and light strength of 350ms in the CA1-CA2.

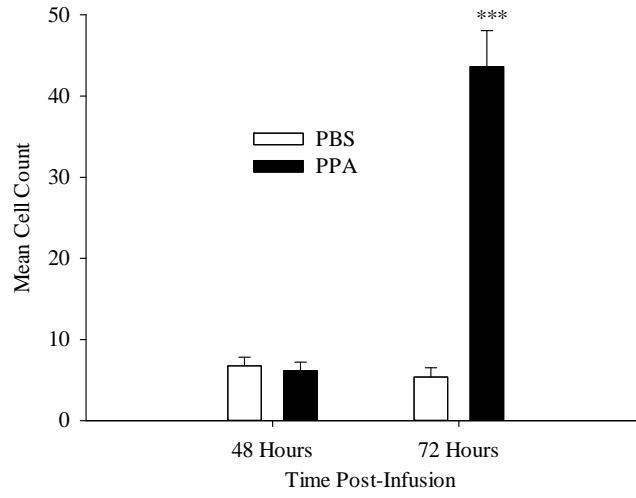
3.3.2.2 Activated Microglia (CD68)

The 2x2 ANOVA revealed a drug by timing interaction of microglial activation in the dentate gyrus ($F(1,27) = 61.14, p < 0.001$) and the CA1-CA2 ($F(1,27) = 27.18, p < 0.001$). In contrast, the white matter did not present with activated microglia (drug: $F(1,27) = 3.95, ns$, timing: $F(1,27) = 0.04, ns$, interaction: $F(1,27) = 0.12, ns$) (see Fig. 3.10C) nor did the cortex (drug: $F(1,27) = 0.13, ns$, timing: $F(1,27) = 0.07, ns$, interaction: $F(1,27) = 0.003, ns$) (see Fig. 3.10D). MANOVA and Tukey's posthoc revealed that the mean microglial cell count was greatest at 72 hours following PPA infusion (Dentate gyrus: $F(3,27) = 58.49, p < 0.001$; CA1-CA2: $F(3,27) = 33.93, p < 0.001$). These results are shown in Figure 3.10A-3.10B with qualitative evidence in Figure 3.10E.

Acute and spaced infusion schedules were compared at 48 hours and there were no significant differences in drug ($F(1,27) = 0.66, ns$), timing ($F(1,27) = 1.19, ns$) or their interaction ($F(1,68) = 1.78, ns$) in the dentate gyrus (Fig. 3.11A). In the CA1-CA2 however, a main effect of infusion frequency was found ($F(1,27) = 8.29, p < 0.01$) such that 4 infusions had greater microglial activation than a single infusion. There was no main effect of drug ($F(1,27) = 0.50, ns$) or interaction ($F(1,27) = 0.001, ns$) (Fig. 3.11B). This pattern was also seen in the white matter with a main effect of infusion frequency ($F(1,27) = 21.85, p < 0.001$) and a non-significant main effect of drug ($F(1,27) = 0.70, ns$) and drug by infusion frequency interaction ($F(1,27) = 2.84, ns$) (Fig. 3.11C). Therefore CD68 cell counts were significantly greater with 4 infusions, regardless of drug. See Figure 3.13 for qualitative view of microglial activation in the CA1-CA2.

At 72 hours post-infusion, there was evidence of microglial activation in the dentate gyrus (Fig 3.12A) and the CA1-CA2 (Fig. 3.12B). A drug x infusion frequency interaction was shown (dentate gyrus: $F(1, 27) = 10.28, p < 0.01$, CA1-CA2: $F(1,27) = 11.47, p < 0.01$) such that four infusions of PPA produced significantly greater activation than all of the other groups ($p < 0.001$). Additionally, 1 infusion of PPA had greater cell counts than its associated control ($p < 0.001$). In contrast, microglial cell counts in the white matter (Fig. 3.12C) were not different based on drug ($F(1,27) = 0.02, ns$), number of infusions ($F(1,27) = 3.82, ns$), or its interaction ($F(1,27) = 1.10, ns$).

A- CD68 Immunopositive Staining in CA3/Dentate Gyrus



B- CD68 Immunopositive Staining in CA1/CA2

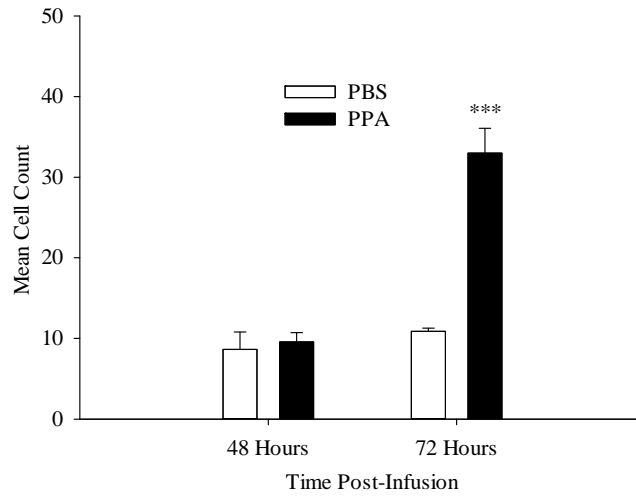
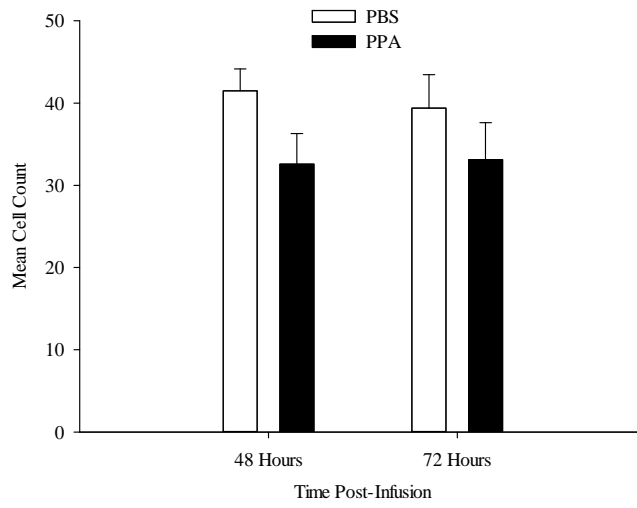


Figure 3.10: CD68 staining across 2 different perfusion time points

C- CD68 Immunopositive Staining in the White Matter



D- CD68 Immunopositive Staining in the RSGb Cortex

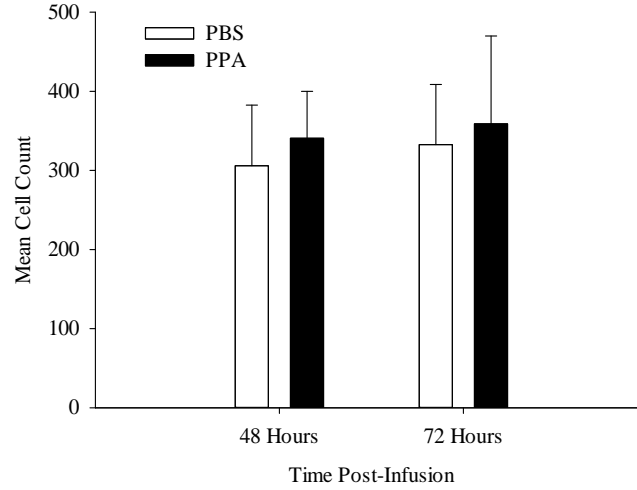


Figure 3.10 cont.: CD68 staining across 2 different perfusion time points

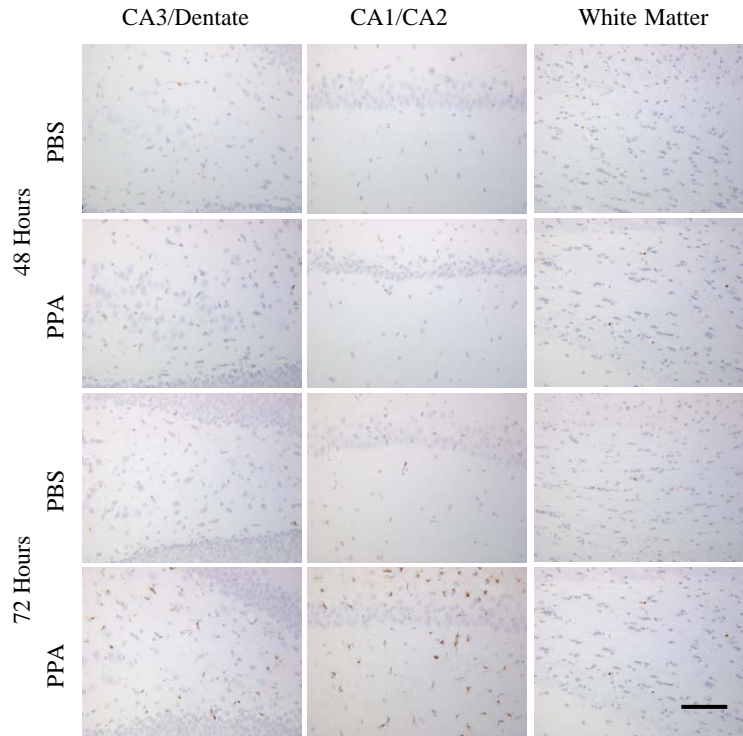
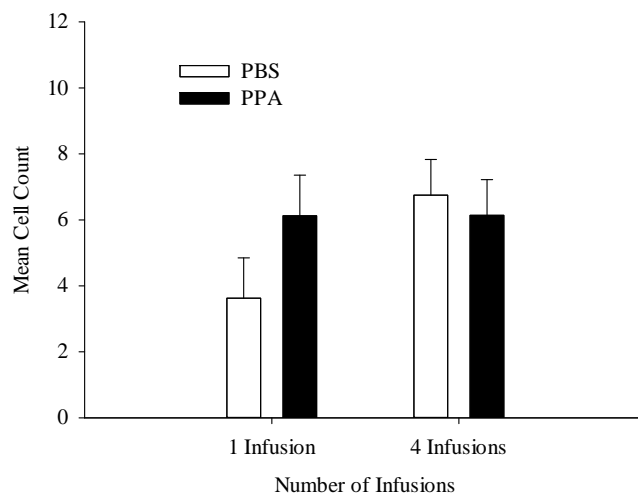


Figure 3.10: CD68 staining across 2 different perfusion time points and two drug groups (N = 8 for all groups except PPA/48hours where N = 7). Cell counts were taken in the dentate gyrus (A), CA1-CA2 (B), white matter (C) and cortex (D). Note the change of scale in (D). Images representing individual microglia are arranged by brain region, timepoint and drug (E). Each bar indicates group mean data across drug and perfusion time point. Error bars represent +SEM. *** $p < 0.001$, 72 hours after PPA infusion significantly greater than all other treatment groups. (Scale bar is equal to 100 μ m)

A- CD68 Immunopositive Staining in CA3/Dentate Gyrus at 48 Hours



B- CD68 Immunopositive Staining in CA1/CA2 at 48 Hours

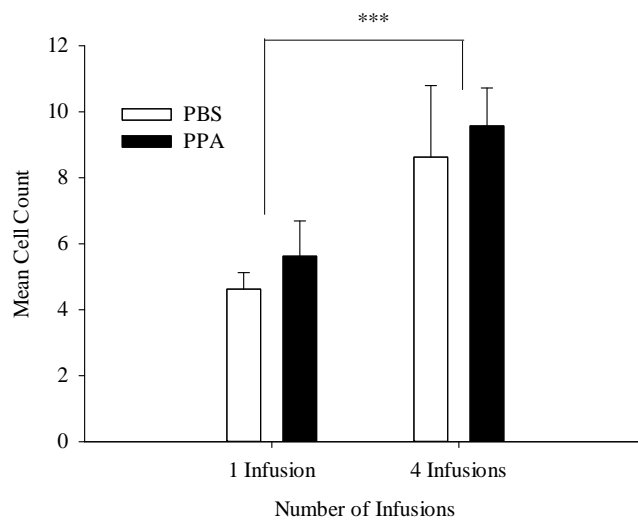


Figure 3.11: CD68 cell counts comparing 1 infusion to 4 infusions at 48 hours

C- CD68 Immunopositive Staining in the White Matter at 48 Hours

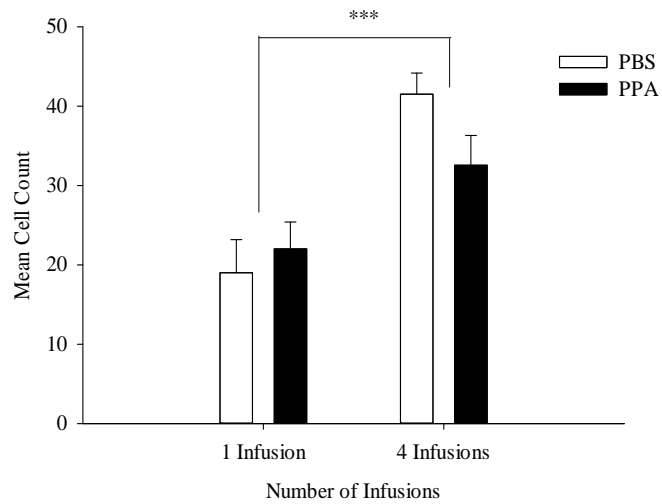
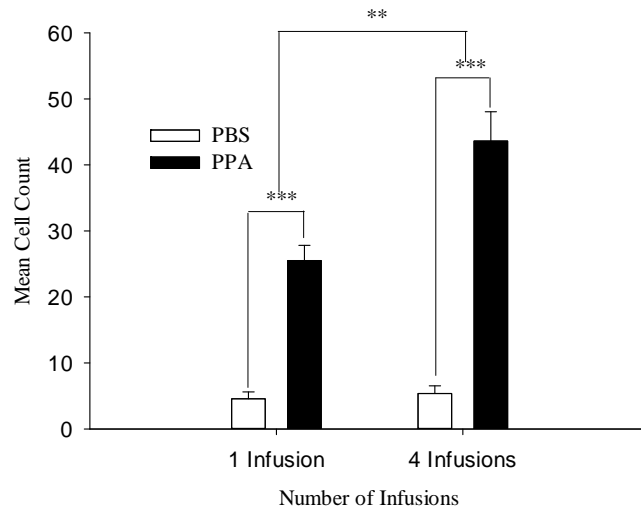


Figure 3.11 cont.: CD68 cell counts comparing 1 infusion to 4 infusions at 48 hours post-infusion of PBS (N = 8 for both groups) or PPA (N = 8 for 1 infusion, N = 7 for 4 infusions) in the dentate gyrus (A), the CA1-CA2 (B) and the white matter (C). Note change in scale in (C). Each bar indicates group mean data across drug and perfusion time point. Error bars represent +SEM. *** $p < 0.001$, 4 infusions greater cell count than 1 infusion

A- CD68 Immunopositive Staining in CA3/Dentate Gyrus at 72 hours



B- CD68 Immunopositive Staining in CA1/CA2 at 72 Hours

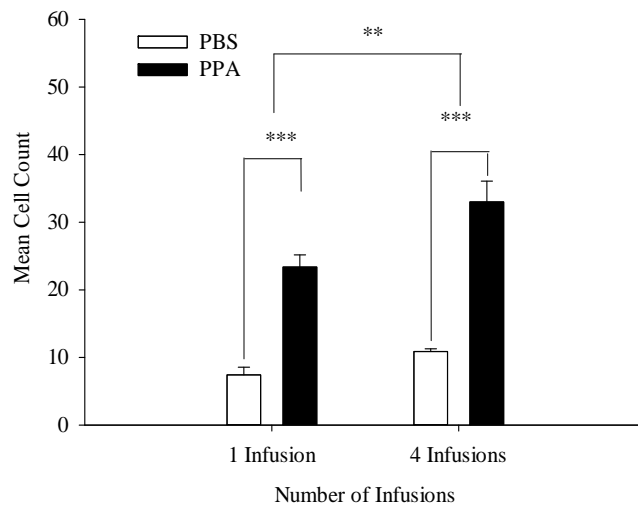


Figure 3.12: CD68 cell counts comparing 1 infusion to 4 infusions at 72 hours

C- CD68 Immunopositive Staining in the White Matter at 72 Hours

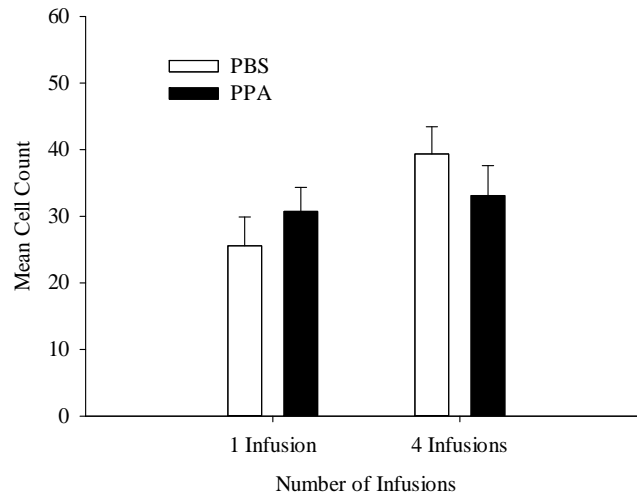


Figure 3.12 cont.: CD68 cell counts comparing 1 infusion to 4 infusions at 72 hours post-infusion of PBS (N = 7 for 1 infusion, N = 8 for 4 infusions) or PPA (N = 8 for both groups) in the dentate gyrus (A), the CA1-CA2 (B) and the white matter (C). Each bar indicates group mean data across drug and perfusion time point. Error bars are +SEM. *** $p < 0.001$, PPA greater than PBS, ** $p < 0.01$, 4 Infusions of PPA greater than all of the other treatment groups.

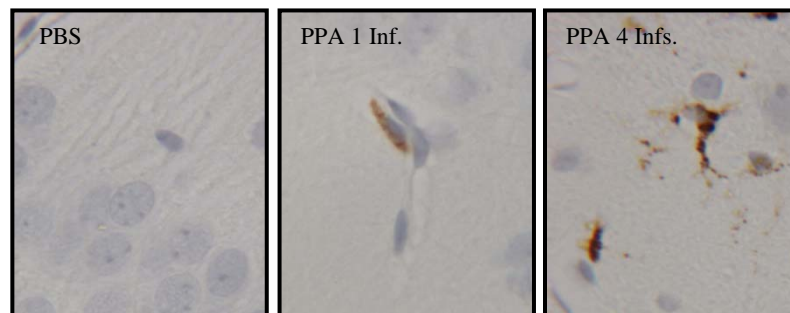


Figure 3.13: Qualitative view of CD68 following infusion of PBS or PPA. PPA 1 inf. labels image of microglial activation at 72 hours following single ICV infusion and PPA 4 infs. labels image of microglial activation at 72 hours following four spaced ICV infusions. Images were taken at an objective power of 63 and exposure of 350ms in the CA1-CA2.

3.4 Discussion

The current findings revealed that temporally spaced and repeated PPA infusions affect locomotor activity and neuroinflammatory responses. Increased total distance traveled and mean velocity were displayed for PPA treated animals relative to controls, and astrogliosis and microglia activation were prominently featured. When compared to a single infusion, the spaced treatment of one infusion a week for four weeks induced significantly increased microglial proliferation at 72 hours after the final infusion and delayed the astrocytic reaction by 24 hours.

3.4.1 Behavioural Parameters

Repeated weekly ICV infusions of PPA resulted in a significant effect of drug for total distance traveled and mean velocity. PPA-infused animals traveled significantly further and faster than control animals irrespective of the week evaluated. With repeated infusions of PPA, it was interesting that animals did not develop a tolerance or sensitization to its effects. In particular, locomotor sensitization has been commonly reported with chronic administration of ethanol and amphetamines (Fukushiro et al., 2012), where animals show an enhancement in motor behaviours. Additionally, behavioural tolerance is common upon repeated exposure to LPS, and cytokines are thought to mediate this process (Cavaillon, Marie, Pitton, & Fitting, 1995). It is thus possible that PPA-induced signaling cascades that modulate cytokine production are not diminished, perhaps as a result of low-grade inflammation development (see Chapter 2 Discussion for an overview of additional fast-acting mechanisms). Similarly, it is possible that PPA treated animals fail to recognize the familiarity of the open field, suggesting possible learning and memory deficits.

Analyses of locomotor activity 48 and 72 hours after the final infusion revealed no significant differences between the PPA treated and PBS infused control groups. Overall, four PPA infusions triggered the rats to move further and faster as compared to controls, however these responses were diminished following 48 and 72 hours drug-free.

In contrast to the findings of Chapter 2, four spaced ICV infusions of PPA did not affect the duration of time spent in the centre zone and there were no differences when evaluated after 2-3 drug-free days. Previous studies evaluating thigmotaxis in an open

field have reported similar findings. Benzaquen et al., (2010) found that PPA treated animals consistently exhibited increased anxiety-like behaviours following IP injections as measured in the VersaMax apparatus, but not in a novel open field. Similarly Shams et al. (2009) examined thigmotaxis behaviour using Ethovision and VersaMax and found that VersaMax was a more sensitive measure. It is therefore possible that methodologically, an open field is not a valuable measure for examining PPA-induced anxiety effects. Considering the PBS control animals also spent less than 50% of the duration in the centre zone, which would equate to 600 seconds (see Fig. 3.2C), the novelty of the apparatus may be an important factor worth exploring. In general, thigmotaxis is used to measure anxiety-like behaviours in a novel environment (Treit & Fundytus, 1988) but all of the assessments of activity after the first day of infusion would no longer be in a novel open field. Measuring anxiety in a T-maze or during a light/dark test may clarify the present findings. Additionally small group sizes following reassessment of activity may be insufficient for analysis.

An alternative explanation would be that chronic infusions of PPA do not affect emotionality or mood, and this has been supported by Brusque et al. (1999). Rats chronically injected systemically with PPA did not display anxiogenic behaviours, allowing the researchers to infer that chronic injections of PPA do not promote anxiety-like movements. Because these are systemic effects of gut metabolites, it is necessary to further investigate PPA's central role in anxiety-related behaviours considering the current findings indicate that PPA has little effect on thigmotaxis.

3.4.2 Immunohistochemistry

Within the dentate gyrus, hippocampus and white matter PPA treated rats exhibited significantly increased astrocytic mean area 72 hours after the final infusion.

Qualitatively, the astrocytes' morphology exemplified stage 2 of activation: severe diffuse reactive astrogliosis (see Fig. 3.9 panel 3; Sofroniew & Vinters, 2010). This category is characterized by extensive overlapping processes and typically results in long-lasting organizational modifications. Accordingly, severe reactive astrogliosis is presumed to be neurotoxic at the site of injury through the upregulation of IL-6, TNF- α ,

IFN- γ , NO, and TLRs (Sofroniew, 2005) and the downregulation of IL10, GSH (Chen & Swanson, 2003), and ATP (Chakraborty, Kaushik, Gupta, & Basu, 2010).

The absence of an effect in the RSGb cortex also coincides with the proposed stage of severe diffuse astrogliosis. A defining feature of reactive diffuse astrogliosis is that pro-inflammatory cytokines are produced within the immediate vicinity of the injury, assumed to be the hippocampus (Sofroniew & Vinters, 2010), but anti-inflammatory effects are notable in adjacent tissue, such as the cortex. Again it would be worthwhile to determine cytokine levels in the CNS to support these findings.

In general astrocytes are more resistant to changes in the microenvironment relative to neurons (Kirchhoff, Dringen, & Giaume, 2001) with the exception of acidosis (Lukaszevicz et al., 2002) and oxidative stress (Hollensworth et al., 2000). With chronically low pH, the sodium bicarbonate cotransporter on astrocytes induces an inward flow of sodium, resulting in intracellular acidosis (Giffard, Monyer, & Choi, 1990; Giffard et al., 2000). PPA is known to induce changes in sodium transporters (Wyse et al., 1998), and so does possess the means to influence the CNS pH, thereby inducing activation of astrocytes. Because neurons are not as affected by fluctuations in pH as compared to astrocytes, if intracellular acidification is in fact an underlying contributor of PPA induced neuroinflammation, the absence of neuronal cell death in the PPA animal model (MacFabe et al., 2007) is not unexpected. Qualitatively, there was no evidence of gross neuronal losses in the current study, however this is an avenue worth exploring. Most reports evaluating the relationship between toxins or CNS trauma and astrocytic activation, have noted that astrogliosis tends to precede neuronal cell death (Chen & Swanson, 2002). It's possible that the employed timeline does not account for the evaluation of cell death however it is important to acknowledge that typically ASD is not accompanied by neuronal losses (Pardo & Eberhart, 2007; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005).

As reviewed by Sofroniew & Vinters (2010), astrocytes can undergo three progressive stages of activation: mild/moderate, severe diffuse astrogliosis and severe astrogliosis with glial scar formation. According to the morphology of the cells and the pattern of

activation, a single infusion of PPA stimulates mild to moderate activation of astrocytes while four spaced infusions results in the severe diffuse reaction (see Fig. 3.9). Not only are the astrocytes qualitatively different across treatment paradigms, the time of activation is earlier with a single ICV infusion. At 48 hours, astrocytes were significantly more hypertrophic with one infusion relative to four, whereas at 72 hours, four spaced infusions of PPA resulted in increased area of astrocytes as compared to one infusion of PPA. The reason for this delay is largely unknown although it may be related to receptor adaptation.

Short chain fatty acids can bind to and activate a family of G-protein coupled receptors (GPCR) that are commonly found spanning the membrane of inflammatory cells, and in the gastrointestinal tract (Brown et al., 2003; Maslowski et al., 2009). GPCR43 are targeted primarily by propionate and acetate and once bound are activated in a dose-dependent manner (Brown et al., 2003). Endogenous ligand binding can induce the receptors to increase intracellular calcium in glial cells (Le Poul et al., 2003), resulting in their activation. When repeatedly stimulated, adaptation can occur such that the receptor doesn't produce the same intensity of depolarization until the stimulus intensity is changed (Sherwood & Kell, 2010). With repeated spaced infusions of PPA, glial cells could have been maintained at low-grade activation until the removal of PPA was detected in which case a delay in astrogliosis would occur. Although GPCR43 physiology is only now becoming elucidated it is possible that these receptors may be therapeutic targets considering their link between gut-associated inflammation and central inflammation (Bindels, Dewulf, & Delzenne, 2013; Kim, Kim, & Kwak, 2012)

Immunohistochemical analysis of activated microglia, using CD68 as a molecular marker, revealed significant increases in cell proliferation in the dentate gyrus and hippocampus 72 hours after the final infusion of PPA. In contrast, there were no significant effects in the white matter or cortex indicating that microglial proliferation is distinctly localized. The earlier suggestion that grey matter may be initially targeted by PPA is refuted by the finding that the images spanning the RSGb cortex did not demonstrate increased microglial activation relative to controls. It is interesting, however that the proliferation of immunopositive cells in the cortex is considerably more extensive than in the hippocampus, dentate gyrus, and white matter. Upon closer examination of the tissue it

was shown that the implanted cannula directly penetrates the cortex and therefore the high level of microglial activation likely results from the trauma of the cannula to the RSGb cortex and is not dependent on drug infusion.

Qualitatively, the PPA activated microglia have a different morphology as compared to PBS treated animals such that there are shorter processes and reduced branching. Round microglia cells are considered to represent the end stages of microglia activation, which may indicate that the repeated infusion paradigm resulted in attainment of macrophage morphology (Ovanesov et al., 2006). In order for this to be determined, biomarkers of phagocytes/macrophages should be investigated.

The finding that microglial proliferation was elevated at 72 hours in the brains of PPA-injected animals, regardless of the number of injections, aligns with the proposal that microglia activation may be an all-or-nothing response that occurs irrespective of the nature of the pathology (Gerhmann et al., 1995). It is suggested that once a certain level of toxicity is reached, microglia immediately become active. In this respect, although there are specific stages necessary for activation attainment (Gerhmann et al., 1995), the process may not be as graded as originally believed, or the degree of proliferation may be dose-dependent after obtaining a basal level of toxicity. This latter scenario would be well supported by the finding that the repeated/spaced PPA regime induced significantly more proliferation at 72 hours than the single injection paradigm in the dentate gyrus and the hippocampus (see Fig. 3.12).

Likewise, the reaction resulting from one infusion may simply represent activated but not phagocytic microglia, whereas with four PPA injections the microglia may be phagocytic cells. The particular stain (CD68) used in these experiments does not differentiate between phagocytic and non-phagocytic cells, and so future studies should evaluate downstream markers of phagocytes such as the presence of hydrogen peroxide (Colton & Gilbert, 1987) and nitric oxide (Banati, Rothe, Valet, & Kreutzberg, 1993). Similarly phagocytic cells are known to become hypertrophic with large somas and short processes (Montero-Menei et al., 1996) and so size differences could provide meaningful information. These suggestions are highly speculative however and require further investigation.

Tanaka et al. (2006) evaluated the qualitative differences in microglia between a single central infusion of LPS and those of a 5 day infusion schedule. In their study, the cannula was placed directly into the hippocampus and it was shown that within 2 hours of a single infusion microglia were immunoreactive for CD11 β , which highlights both macrophages and activated microglia. Expression of IL-1B and TNF- α cytokines were also rapidly upregulated with strong immunoreactivity by 6 hours. Similarly the long-term activation of microglia was shown in the animals treated with LPS for 5 days. Interestingly the cytokine expression was colocalized with microglia but not astrocytes suggesting that microglia produce IL-1B and TNF- α directly. Likewise IL-1B induces iNOS, which as previously described can lead to NO release and a host of insults. Although quantitative evidence for activation was not provided, these researchers were able to show that the increased microglial activation from 5 days of treatment coincided with learning and memory deficits but not locomotor changes. Recall that within the same time frame of microglia activation, there are no differences in total distance traveled, mean velocity or thigmotaxis. This further exemplifies why the evaluation of different behaviours is necessary as bacterial products can induce immune responses, which appear to directly affect hippocampus related neural functions. Additionally, examining earlier time points are necessary.

3.4.3 Temporal Relationship

Analysis of variances revealed that with 4 spaced infusions of PPA, microglia and astrocytes were both activated 72 hours following the fourth and final ICV infusion. Further investigation also found that with a single PPA infusion, astrogliosis was significantly increased at 48 hours compared to four infusions, although this trend was reversed at 72 hours. Specifically, comparing levels of astrogliosis between one infusion and four infusions three days after PPA exposure revealed that the repeated treatment regime resulted in significantly more astrogliosis. The difficulty in interpreting these results lies in the constraints of including only two time points, as it is possible that glial cell activation may begin prior to 48 hours and persist beyond 72 hours. Previous findings from the PPA animal model have found that 24 hours after ICV infusions, immunomarkers were visible (MacFabe et al., 2007; 2008; Shultz et al., 2008).

Shultz and colleagues (2008) used an intermediate schedule with one central infusion of PPA a week for two weeks. Rodents were perfused 24 hours following the final injection, and neuropathology findings indicated a statistically significant elevation of astrocytes' mean area in the hippocampus only. The number of GFAP immunopositive cells were also evaluated and found to be similar to controls. According to the stages of activation described by Sofroniew and Vinters (2010) this would be categorized as mild to moderate astrogliosis, as there was no evidence of diffuse activation and qualitatively, the astrocytes were shown to have individual domains with no overlapping processes. These results demonstrated that astrogliosis can occur as early as 24 hours following two spaced infusions and that the hippocampus may be the initial point of PPA-induced injury. Similarly, after two spaced infusions, CD68 cell counts were not significantly increased at 24 hours. These results not only confirm the current findings that hypertrophic processes and not proliferation of astrocytes characterize the primary stages of astrogliosis, but suggest that a 24 hour time point would be meaningful in spaced injection paradigms. If an additional perfusion time point were to be added to the treatment regime of four spaced infusions, and astrogliosis was evident, this could clarify the role of astrocytes in PPA neuroinflammation. Similarly, extending the timeline past 72 hours in order to evaluate the permanence of the reaction could facilitate the understanding of the molecular underpinnings.

3.4.4 General Conclusions

The findings of the current study coincide with those of Chapter 2 in that PPA produced modifications in locomotor behaviour and innate inflammatory markers. The distinction between an acute and repeated infusion paradigm was found to be that astrocyte activation was slightly delayed after 4 infusions. Microglia activation was found to be dose-dependent as four ICV infusions produced significantly greater proliferation than one.

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Chapter 4

General Discussion

The temporal relationships between locomotor activity, astrogliosis and microglial activation were ascertained in both an acute and repeated infusion paradigm using propionic acid (PPA). With one intracerebroventricular (ICV) infusion of PPA, rats exhibited significant increases in total distance traveled, mean velocity and total duration in a centre zone: thigmotaxis, within 30 minutes of infusion. These behaviours were transient however, as analyses revealed no differences between PPA treated animals and controls when evaluated after 2-3 drug-free days. In contrast, astrogliosis and microglial activation obtained maximal values 48 and 72 hours after a single PPA infusion and these immunomolecules had temporally distinct activation times. Activated astrocytes were present at 48 hours and returned to baseline values by 72 hours at which point microglial activation predominated. In contrast, with a repeated treatment schedule of one infusion a week for four weeks, both astrocytes and microglia were activated at 72 hours following the fourth ICV infusion of PPA. Comparisons between acute and spaced/repeated infusions revealed a delay in the activation of astrocytes and a significantly greater microglial response resulting from the spaced PPA paradigm. Behaviourally, rats exposed to repeated ICV infusions of PPA displayed greater distance traveled and mean velocity but no significant effects in thigmotaxis when evaluated immediately following each infusion.

4.1 ASD and Glial Alterations

Although it is unlikely that so few exposures to an environmental toxin can lead to the development of acquired mitochondrial disease (Clark-Taylor & Clark-Taylor, 2004), aberrant immune responses (Jyonouchi, Sun, & Itokazu, 2002; Morgan et al., 2010; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005) and gastrointestinal dysfunction (de Magistris et al., 2010) evident in Autism Spectrum Disorders (ASD), these series of experiments emphasize the efficacy of ICV treatment with PPA in inducing transient neuroinflammation. Although numerous etiologies of ASD have been proposed, some researchers believe that these all converge to a common pathway with the suggestion that central and peripheral inflammation is a likely underlying factor (Depino, 2013). The idea that early pre- or post-natal bacterial and/or viral infections contribute to ASD is supported by rodent studies of maternal immune activation (Garay, Hsiao, Patterson, & McAllister, 2012; Malkova, Yu, Hsiao, Moore, & Patterson, 2012;

Patterson, 2011). However only a few studies have evaluated the relationship between postnatal infection and ASD in humans (Depino, 2013). The findings of the current study further support that bacterial metabolites may contribute to ASD etiology. Specifically it was found that PPA mediates neuroinflammation in adult rodents. In particular, these results suggest that activated glial cells (astrocytes and microglia) may contribute to the deficits seen in ASD.

PPA is a primary energy source for astrocytes, and once metabolized to propionyl CoA and methymalonic-CoA can directly influence mitochondrial function (Grunert et al., 2013; Al-Lahham, Peppelenbosch, Roelofsen, Vonk, & Venema, 2010). Specifically as reported by Frye, Melnyk, & MacFabe (2013), PPA causes significant alterations within the citric acid cycle. When PPA is metabolized it enters the Krebs cycle as succinyl-CoA, which if elevated decreases the high-energy electron carrier ratio (NADH:FADH₂) from 3:1 to 1:1. This directly reduces ATP production from the electron transport chain. As a consequence of diminished ATP, neuronal and astrocytic functions are compromised and irreversible injury occurs (Chen & Swanson, 2003).

Likewise Palmieri & Persico (2010), have also suggested that mitochondrial disorder may underlie ASD. Through abnormal immune regulation and cytokine activation, intracellular calcium spikes become enhanced and irregular, which can directly impact energy metabolism and consequently produce multiple reactive oxygen species. These can induce mitochondrial dysfunction and subsequent oxidative stress resulting in abnormal synaptic functioning. In this pathogenic model, glial cells can be implicated as 1) initiating the cascade through the production of cytokines, or microglia specifically; or 2) being a secondary response to already increased calcium and altering synaptic plasticity. Abnormal brain circuitry can directly lead to the behavioural and cognitive deficits typical of ASD (Rodriguez & Kern, 2011). PPA is an important ligand for two classes of G-protein coupled receptors: GPCR41 and GPCR43, and GPCR43 are present on immune molecules such as astrocytes and microglia (Bindels, Dewulf, & Delzenne, 2013). The notion that glial cells are a secondary response to altered calcium is supported by the fact that when PPA is bound to GPCR43, a signaling cascade is induced such that intracellular calcium levels are increased (Le Poul et al., 2003).

Analogous to the findings in Chapter 2, small increases in central PPA can cause perturbations that result in the activation of astrocytes and subsequently of microglia in the hippocampal area. As microglia become activated, the overexpression of chemokines and cytokines propagates the inflammatory response (Gehrmann, Matsumoto, & Kreutzberg, 1995). In Chapter 3, evidence is provided that increased amounts of PPA produce greater microglia activation and astrogliosis, however further examination is required in order to determine whether changes are more prolonged and persistent. As well, whether similar alterations exist in other brain areas need to be evaluated.

While metabolic dysfunction may trigger the delayed inflammatory response, more fast-acting mechanisms likely contribute to the locomotor abnormalities with subsequent activation of inflammatory markers. Hyperactivity and anxiety are common findings in ASD (Theoharides, Asadi, & Patel, 2013), and a variety of neurotransmitters have been frequently implicated in modulating movement and mood (Forsythe, Sudo, Dinan, Taylor, & Bienenstock, 2010). The behavioural findings of the current series of studies may then also be explained by altered neurotransmitter synthesis and/or release. The effects of PPA on neurotransmitter systems include increasing glutamate through NMDA receptor sensitivity (de Mattos-Dutra et al., 2000) and decreasing levels of GABA, dopamine and serotonin as determined in brain homogenates of rodents exposed to systemic PPA (El-Ansary, Ben, & Kotb, 2012). When serotonin and dopamine neural circuits are dysregulated, such as with the administration of methamphetamine (Lavoie, Card, & Hastings, 2004), the delayed activation of astrocytes and microglial is enhanced and the temporal relationship coincides with that of the current studies.

In general it is likely that astrogliosis and microglial proliferation are secondary to PPA's effects on NMDA receptor activity (de Mattos-Dutra et al., 2000), intracellular calcium levels (Nakao, Fujii, & Niederman, 1992), gap junction functioning (Rorig, Klaus, & Sutor, 1996), oxidative phosphorylation (Brass & Beyerinck, 1988), and cytokine release (Cavaglieri et al., 2003). This might explain why glial activation occurs two to three days following infusion, while locomotor changes are rapid.

A suggested mechanism for a more long-term effect may then be that PPA induces abnormal levels of neurotransmitters produced from neurons which overwhelms the

abilities of astrocytes to remove them leading to motor behaviour abnormalities, excitotoxicity and increased production of cytokines and chemokines. Subsequently these chemical messengers recruit monocytes to areas of insult and microglia become activated and eventually phagocytic. This proposal is supported by the finding that PPA influences the NA/K pump and that sodium transporters on the astrocytes are essential for glutamate clearance (Anderson & Swanson, 2000). This potential pathway would be important for one injection of PPA and four spaced infusions, where microglia and astrocytes act collectively to mediate neural transmission. In fact, microglia have been considered to play a fundamental role in NMDA receptor neuronal injury and sensitivity (Gehrmann et al., 1995). Interestingly under settings of low ATP production, which has been previously described as a direct consequence of PPA-induced metabolic dysfunction (Frye et al., 2012), astrocytes and neurons increase glutamate efflux further altering the environment of the CNS (Longuemare & Swanson, 1995). Biomarkers found in the plasma of ASD patients are similar to those of patients possessing disorders of glutamate toxicity (Pastural et al., 2009).

4.2 Future Directions

There are a variety of directions in which future research on the effects of PPA could proceed. One particularly interesting proposal would be to infuse a PPA antagonist and decipher the timeline of behavioural and brain changes. Once administered, the antagonist may prevent astrogliosis and microglial activation through its interaction with GPCR43, which would indicate that activation of the G-protein cascade is fundamental to the production of PPA-induced inflammation. It would be interesting to see whether locomotor activity, as well as other behaviours, are affected, so that the function of immune responses in behavioural changes can be deciphered. In particular, further evaluations of different anxiety measures and learning/memory related behaviours are necessary.

Note that the term PPA-induced neuroinflammation has been used in the previous studies to exclusively define the presence of astrocytes and microglia. In reality, neuroinflammation is classified by the expression and presence of numerous molecules such as iNOS, IL-1, IL-6, TNF- α , NF- κ β (O'Callaghan, Sriram, & Miller, 2008). In order

to be confident that central infusions of PPA produce aberrant immune responses, these biomarkers need to be evaluated. Similarly, additional time points should be included to evaluate earlier immune activation with repeated infusions and persistent changes with one infusion. Extending the timeline and analyzing other behavioural parameters would also clarify the relationship between neuroinflammation and behavioural changes.

Because the premise of the animal model is that abnormal amounts of PPA are produced by the gut, these two experiments should be repeated with systemic administrations and in adolescents. Likewise, as ASD is a developmental disorder, it would be imperative to evaluate PPA effects prenatally. Currently, we are feeding mothers diets that are high in PPA and evaluating their offspring. Preliminary pathology findings reveals altered synaptogenesis, increased neuronal density, enhanced astrogliosis and decreased GABAergic neurons (Taylor et al., 2013). Overall, the current studies have provided preliminary evidence for PPA as an important mediatory of locomotor activity and innate inflammation in an animal model of ASD.

4.3 References

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Appendices

Appendix A: Ethics Approval for Animal Use



AUP Number: 2008-063 **PI Name:** Macfabe, Derrick **AUP Title:** Neurobiology Of Enteric Short Chain Fatty Acids In Health And Disease

Approval Date: 02/11/2013

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Neurobiology Of Enteric Short Chain Fatty Acids In Health And Disease " has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2008-063::5

- 1 This AUP number must be indicated when ordering animals for this project.
- 2 Animals for other projects may not be ordered under this AUP number.
- 3 Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura on behalf
of the Animal Use Subcommittee
University Council on Animal Care

The University of Western Ontario
Animal Use Subcommittee / University Council on Animal Care
Health Sciences Centre, • London, Ontario • CANADA – N6A 5C1
PH: 519-661-2111 ext. 86768 • FL 519-661-2028
Email: auspc@uwo.ca • <http://www.uwo.ca/animal/website/>

Stacey Holbrook

ACADEMIC BACKGROUND	<p>MSc. Neuroscience 2011-2013 University of Western Ontario</p> <ul style="list-style-type: none"> • Thesis Title: The effects of a single acute and repeated intracerebroventricular infusions of propionic acid on locomotor activity and neuroinflammation in rats <ul style="list-style-type: none"> ○ <u>Supervisors:</u> Dr. Klaus-Peter Ossenkopp, Dr. Martin Kavaliers, & Dr. Derrick MacFabe <p>B.Sc. (Hons.) Double Major in Medical Sciences /Psychology 2006-2011 University of Western Ontario</p> <ul style="list-style-type: none"> • <u>Independent Research Title:</u> Behavioural and neuropathological effects of a single intracerebroventricular injection of propionic acid: Implications for an animal model of Autism <ul style="list-style-type: none"> ○ <u>Supervisors:</u> Dr. Klaus-Peter Ossenkopp, & Dr. Derrick MacFabe
PROFESSIONAL & ACADEMIC AWARDS	<p>Ontario Graduate Scholarship 2012-Present Value: \$15,000</p> <p>Western Graduate Research Scholarship 2012-Present</p> <ul style="list-style-type: none"> • Value: \$7,000 <p>Western Graduate Research Scholarship 2011-2012</p> <ul style="list-style-type: none"> • Value: \$7,200 <p>Dean's Honour List 2008-2011</p>
ACADEMIC & TEACHING EXPERIENCE	<p>Teaching Assistant- Topics in Behavioural & Cognitive Neuroscience 2013</p> <p>Graduate Level Course in Statistics 2012</p> <ul style="list-style-type: none"> • Analysis of Variance, Hierarchical Linear Modelling, Multiple Regression & Correlation, Factor Analysis, R programming <p>Teaching Assistant- Research Methods and Statistical Analysis 2011-2012</p> <ul style="list-style-type: none"> • Nominated for CCDP Teaching Assistantship Award <p>Research Assistant Position 2009-2011</p> <ul style="list-style-type: none"> • Kilee Patchell-Evans Autism Research Group <ul style="list-style-type: none"> ○ Conducted research focusing on the development of an animal model of autism
TECHNICAL & SPECIALIZED	<p>Animal Care and Veterinary Services Certification</p> <ul style="list-style-type: none"> • WebCT Animal Care & Use Course

SKILLS

- Basic Rat training
- Rat Surgery Part 1 & 2
- Experience with intraperitoneal and subcutaneous injection techniques
- Perform intracerebroventricular surgery on rats

PUBLICATIONS-

POSTER PRESENTATIONS

Holbrook, S., Boon, F., Taylor, R., Thomas, R., Tichenoff, L., Ossenkopp, K.-P. and MacFabe, D.F. Temporal effects of single intracerebroventricular infusions of Propionic Acid on Behavioural, Neuropathological and Lipid changes in rats: Further development of a rodent model of Autism. Program No. 151.09. 2011 Neuroscience Meeting Planner. Washington, DC: Society for Neuroscience, 2011. Online.

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Holbrook, S., Mepham, J. R., Boon, F., Taylor, R., Thomas, R., Kavaliers, M. I., Ossenkopp, K.-P. and MacFabe, D.F. Time course of both single and repeated intracerebroventricular infusions of Propionic Acid on Behavioural, Cognitive, and Neuropathological changes in rats: Further development of a novel rodent model of Autism. Program No. 444.09. 2011 Neuroscience Meeting Planner. New Orleans, LA: Society for Neuroscience, 2012. Online.

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