

2016

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### Recommended Citation

Strasser, L. (2016). The Effects of Propionic Acid on Locomotor, Repetitive and Anxiety-Related Behaviours in Female Adolescent Rats. *Western Undergraduate Psychology Journal*, 4 (1). Retrieved from <http://ir.lib.uwo.ca/wupj/vol4/iss1/10>

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## The Effects of Propionic Acid on Locomotor, Repetitive and Anxiety-Related Behaviours in Female Adolescent Rats

Lauren Strasser\*

Autism spectrum disorders (ASD) are a family of neurological disorders classified by impaired communication and social behaviour as well as increased repetitive or perseverant behaviours. Propionic acid (PPA) is an endogenous short chain fatty acid shown to induce behavioural and physiological symptoms reminiscent of ASD in rats. The current study examined the effects of PPA on the behaviours of female adolescent rats, thereby expanding the validity of the PPA animal model of ASD. Female adolescent rats were placed in the hole-board apparatus and locomotor and thigmotaxis activity was recorded for 2 baseline days and 3 treatment days. On treatment days, rats received systemic injections of either Phosphate Buffered Saline (PBS,  $n = 6$ ) or PPA (0.26 M,  $n = 6$ ). Findings indicated that PPA and PBS injected rats did not significantly differ on measures of thigmotaxis, stereotypy or locomotor behaviour. This preliminary study suggests that systemic injections of PPA may not influence repetitive, locomotor and anxiety-related behaviours in female adolescent rats. Further research should be conducted to clarify the sex-specific effects of PPA on females.

Autism spectrum disorders (ASD) are a class of neurodevelopmental disorders characterized by repetitive behaviours as well as impaired social behaviour and communication (Rapin & Tuchman, 2008). Additional symptoms include impaired cognitive and executive function, impaired memory and attention, altered sensory responses, hyperactivity, epilepsy, resistance to change, and mental retardation (National Institute of Mental Health, 2013; Rapin & Tuchman, 2008). The prevalence of ASD is approximately 1 in 88 children (Baio, 2012). This demonstrates a 289.5% increase in ASD prevalence from 1997 to 2008 (Boyle et al., 2011). Males are 4.6 times more likely to be diagnosed with ASD (Baio, 2012). Prevalence of ASD in males, but not females, significantly increased from 2007 to 2012 (Blumberg, 2013).

### Etiology of ASD

ASD is a complex disorder involving genetic, epigenetic and environmental factors.

Previous research using twin studies has shown a moderate to strong genetic basis for ASD (Folstein & Rosen-Sheidley, 2001; Hallmayer et al., 2011; Scherer & Dawson, 2011). Current heritability estimates of ASD are reported as high as 90% (Bailey et al., 1995; Folstein & Rosen-Sheidley, 2001). Monozygotic twins have been shown to have concordance rates of 60% to 92%, relative to 0 to 10% in dizygotic twins (Bailey et al., 1995).

Since monozygotic twins do not show complete concordance in prevalence or severity of ASD symptoms, ASD cannot be explained entirely by genetic factors (Hu, Frank, Heine, Lee & Quackenbush, 2006). In fact, only 10% to 20% of individuals with ASD show an identifiable genetic component (Geschwind, 2011). Genetic predispositions must therefore interact with environmental conditions to generate the observed prevalence and symptoms of ASD. For example, maternal prenatal risk factors may account for the higher concordance rate of ASD in dizygotic twins than in non-twin

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siblings (Patterson, 2012). Many genes implicated in abnormal brain development are turned on during embryotic development, implicating the importance of this period (Voineagu et al., 2011). Environmental risk factors for ASD include prenatal exposure to toxins such as ethanol (Nanson, 1992), thalidomide (Strömmland, Nordin, Miller, Akerström & Gillberg, 1994), terbutaline (Zerrate et al., 2007), valproic acid (Ingram, Peckham, Tisdale & Rodier, 2000; Moore et al., 2000), and maternal rubella infection and other infections (Comi, Zimmerman, Frye, Law & Peeden, 1999).

Alternatively, environmental factors can enhance ASD outcomes by providing methods for treatment and minimizing symptoms. It has been found that rats in enriched environments were able to overcome a wide range of neurological deficits, including those induced in animal models of ASD (Laviola, Hannan, Macri, Solinas & Jaber, 2008; Nithianantharajah & Hannan, 2006; Schneider, Turczak & Przewlocki, 2006). Typical environmental enrichment methods include increased environmental complexity (e.g., higher range of sensory information), environmental novelty (e.g., changing objects or positions), higher physical activity (e.g., running wheel) or social engagement (e.g., greater number of rats per cage) (Nithianantharajah & Hannan, 2006). These environments have been correlated with delayed disease onset, cognitive enhancement, and enhanced cellular plasticity in numerous brain disorders (Pang & Hannan, 2013). In children with ASD, being exposed to an enriched sensorimotor environments (i.e. receiving daily olfactory/sensory information and increased exercise for 6 months), increased cognition and decreased severity of ASD symptoms (Woo & Leon, 2013).

Another environmental influence on ASD symptoms may be diet. Prior research has found augmented ASD symptoms after ingestion of

refined dairy or wheat products. These symptoms decline in severity after implementation of a casein or gluten free diet (Jyonouchi, 2009). Thus, prior research highlights the interaction between genetic predispositions and environmental factors in contributing to the development and presentation of ASD.

### **Animal Models of ASD**

Animal models of ASD are crucial to the study of disorders as they allow for the testing of physiological, behavioural and pathological mechanisms of diseases in ways not ethically or practically viable in humans. Current widely examined animal models of ASD include the prenatal maternal immune activation model and the valproic acid exposure model (Meyer, 2013; Rouillet, Lai & Foster, 2013).

Prenatal maternal immune activation (MIA) animal models induce an immune response in pregnant rodents by injecting polyinosinic:polycytidylic acid [poly(I:C), viral mimetic] or lipopolysaccharide (LPS, bacterial mimetic) during gestation (Harvey & Boksa, 2012; Meyer 2013; Patterson, 2012). These agents activate toll-like receptors which cause an inflammation response and release pro-inflammatory cytokines (Akira & Takeda, 2004). Higher ASD risk is associated with viral infection in the first trimester and bacterial infection in the second trimester (Atladóttir et al., 2010). MIA in rodents has been associated with numerous behavioural, neurological, and immune system deficits reminiscent of ASD (Harvey & Boksa, 2012). These include decreased selective attention, social behaviour, exploratory behaviour, working memory and cognitive flexibility (reviewed in Meyer, 2013). Rodent offspring displayed decreased social behaviour, ultrasonic vocalization deficits and abnormalities, increased repetitive behaviours, alterations in serotonergic systems and similar cerebellar pathology as ASD patients (Harvey &

Boksa, 2012; Malkova, Yu, Hsiao, Moore & Patterson, 2012; Shi et al., 2009). One recent study showed that pre-natal and postnatal treatment with combined LPS and PPA caused developmental delay, increased anxiety-related behaviours, and altered acoustic behaviours in adolescent rats (Foley, 2013). This study found male rats, but not female rats, to show alterations in social and locomotor behaviour; thus implying a potential sex-difference in the MIA model. This may be due to proven differences in each individual sex's central nervous system's (CNS) response to prenatal infection (Boksa, 2010; Wang et al., 2010). Thus, sex may be a potential modulating factor between MIA and CNS disorders, such as ASD.

Valproic acid (VPA) is an anticonvulsant and mood stabilizer often used in the treatment of epilepsy and bipolar disorder. VPA exposure during the first trimester of pregnancy has been found to significantly increase risk of ASD in offspring (Roullet et al., 2013). Rasalam et al. (2005) found that sodium valproate was the drug most commonly associated with ASD, with 8.9% of VPA-exposed offspring developing ASD. VPA animal models of ASD have demonstrated impaired social behaviour and sensory abilities, as well as increased repetitive behaviours and locomotor activity (Schneider & Przewlocki, 2005). This VPA animal model demonstrates significant face validity for numerous core symptoms of ASD in humans (Roullet et al., 2013). For instance, in humans, prenatal VPA exposure has led to increased ASD symptoms such as language impairment, social difficulties, reduced attention, and restricted interests (Bromley, Mawer, Clayton-Smith & Baker, 2008).

Significant sex differences in prenatal exposure to VPA in rodents have been found. Kataoka et al. (2013) found that male, but not female, rats exposed to VPA displayed reduced social behaviour. Schneider et al. (2008) found that male offspring showed greater behaviour

and molecular impairments, while female offspring displayed greater locomotor and repetitive activity. Therefore, VPA may influence behavioural symptoms in a sex specific manner.

### **Gastrointestinal Aspects of ASD**

A subset of ASD patients exhibit gastrointestinal (GI) abnormalities, including chronic diarrhea, constipation, abdominal pain, gastritis, celiac disease, Crohn's disease, colitis, inflammatory bowel disease, and others (Bauman, 2010; Buie et al., 2010). Population rates of GI symptoms in individuals with ASD vary from 9% to 43%, depending on population and definition of GI symptoms (Black, Kaye & Jick, 2002; D'Eufemia et al., 1996). Interestingly, in these populations the severity of GI dysfunction is directly correlated with the severity of ASD symptoms (Adams, Johansen, Powell, Quig & Rubin, 2011). It has been hypothesized that acute GI abnormalities create or enhance behavioural problems and self-injurious behaviour in non-communicative ASD individuals (Buie et al., 2010; Horvath, Papadimitriou, Rabsztyrn, Drachenberg & Tildon, 1999). One explanation for these GI symptoms may be the abnormal gut microflora seen in individuals with ASD (Parracho, Bingham, Gibson & McCartney, 2005).

### **Propionic Acid**

Propionic Acid (PPA) is an endogenous short chain fatty acid (SCFA) that is both an intermediate in cellular fatty acid metabolism and a metabolic end product of enteric acid found in high concentrations in the gut (Al-Lahham, Peppelenbosch, Roelofsen, Vonk & Venema, 2010; Jan et al., 2002). The primary source of PPA occurs naturally from the anaerobic fermentation of undigested food, mainly dietary fibre and resistant starch, in the colon (Cummings, Pomare, Branch, Naylor & Macfarlane, 1987). In addition to endogenous

sources, PPA is commonly used in the fermentation process of dairy products or as a food preservative in refined wheat products (Brock & Buckel, 2004).

**Roles of Propionic Acid.** Propionic acid plays numerous vital roles in metabolic, physiological and neurological function. It can both passively and actively cross the gut-blood and blood-brain barriers allowing for widespread effects throughout the CNS (Karuri, Dobrowsky & Tannock, 1993). PPA and other SCFAs are a significant metabolic energy source within in body and play a vital role as an energy source to the brain during early development (Rafiki, Boulland, Halestrap, Otterson & Bergersen, 2003). PPA and other SCFA have effects on numerous physiological functions including cell signaling, immune function, gene expression, lipid metabolism, and mitochondrial function (MacFabe et al., 2007; Nakao, Moriya, Furuyama, Neiderman & Sugiya, 1998; Parab, Nankova & La Gamma, 2007). PPA crosses the cell membrane leading to intracellular acidification which causes altered neurotransmitter release, gap junction gating and calcium signaling. This leads to impaired neural communication and behaviour (Cannizzaro, Monastero, Vacca & Martire, 2003; Rorig, Klaus & Sutor, 1996; Severson, Wang, Pieribone, Dohle & Richerson, 2003). PPA has been shown to lower fatty acid levels in the plasma and liver, exert immunosuppressant effects, enhance tissue insulin sensitivity, and decrease food intake (Al-Lahham et al., 2010).

**Propionic Acid and Autism.** Elevated PPA production has been correlated with many physiological and neurological symptoms of ASD. *Clostridia*, a gut bacteria shown be elevated in children with ASD, produces PPA through fermentation (Finegold et al., 2002; Parracho et al., 2005). *Clostridial* species have been directly linked to certain ASD symptoms, such as repetitive behaviours, which are reduced when treated with antimicrobials against

*Clostridia* (Bolte, 1998). VPA exposure, which is known to elevate ASD risk, has also been shown to increase levels of PPA and other SCFAs (Ornoy, 2009).

PPA treatment in rats has caused similar brain lipid and acylcarnitine profiles as seen in the blood of patients with ASD (Thomas et al., 2010). PPA-infused rodents have shown innate neuroinflammation (activated microglia and reactive astrogliosis) and oxidative stress profiles consistent with autopsies of ASD patients (MacFabe et al., 2007, MacFabe, Cain, Boon, Ossenkopp & Cain, 2011; Vargas, Nascimbene, Krishnan, Zimmerman & Pardo, 2005). Taken together, these findings imply that PPA may be linked to physiological and neurological aspects of ASD.

Further, these species of bacteria are antibiotic resistant. Antibiotic treatments disrupt protective intestinal microbiota composition allowing for an optimal growth environment for these resistant gut species (Bolte, 1998). Children with ASD were found to be prescribed antibiotics significantly more in their early medical history (Neihus & Lord, 2006; Parracho et al., 2005). These specific resistant gut microflora species, such as *Clostridia*, may influence the pathogenesis of ASD.

Higher levels of these microflora species elevate PPA levels which may produce or augment behavioural symptoms of ASD. For instance, higher levels of PPA caused by the genetic disorder Propionic acidemia, result in numerous symptoms resembling those seen in ASD. These include seizures, stereotyped movements, and developmental/cognitive delays (Feliz, Witt & Harris, 2003). In animal models, intracerebroventricular PPA infusions produce numerous ASD characteristic behaviours, such as repetitive behaviours, restricted interests, impaired social behaviour, increased locomotor activity, impaired sensorimotor ability, and impaired cognition in adult rats (MacFabe et al., 2007, 2011; Shultz et al., 2008, 2009). In male

adolescent rats, MacFabe et al. (2011) found that PPA impaired social behaviour, restricted behavioural interests to specific objects, and impaired cognition demonstrated by failure to adapt to a reversal in the T-maze task. In regards to anxiety-related behaviour, Foley, Ossenkopp, Kavaliers and MacFabe (2014) found that prenatal PPA injections increased anxiety-related behaviours as demonstrated by a decrease in time spent in the centre of a novel open field. This may be due to the rise in concentration of fermentation end products within the body, which has been reported to increase anxiety as measured by the light/dark emergence test (Hanstock, Clayton, Li, & Mallet, 2004). Systemic PPA injections have also shown ASD behavioural symptoms such as impaired social behaviour, increased anxious behaviour, and hypoactivity (Benzaquen et al., 2010; Shams, Kavaliers, Foley, MacFabe & Ossenkopp, 2009). These findings suggest a significant role of PPA in the pathogenesis and behavioural symptoms of ASD. Additionally, findings validate the usage of a PPA animal model for ASD research.

Sex differences within the PPA animal model have rarely been examined. However, when studied, have shown sex-specific effects of PPA. Benzaquen et al. (2010) found that social impairments demonstrated by PPA-infused males were not shown in females. Foley et al. (2014) found that prenatal and postnatal PPA injections caused increased anxiety-like behaviour in female, but not male, rats as measured by the elevated plus maze. Due to robust sex difference in ASD prevalence and sex specific effects found in alternative ASD animal models, further research on sex specific effects of PPA must be examined.

### **Present Study**

The current study examined the effects of PPA on repetitive, locomotor and anxiety-related behaviours in adolescent female rats.

Adolescence has been previously been characterized as a window of vulnerability into psychopathology (Adriani & Laviola, 2003), with exacerbation of many symptoms and clinical deterioration in some ASD patients (Gillberg & Schaumann, 1981; Perisse et al., 2010). In animal studies, adolescents have been reported to show increased levels of social activity (Varlinskaya & Spear, 2002; Varlinskaya & Spear, 2008) and greater susceptibility to the aversive effects of drugs or toxins on the brain relative to other ages (Adriani, Caprioli, Granstem, Carli & Laviola, 2003; Adriani & Laviola, 2003).

Research into potential sex differences in ASD models is crucial as it may help to explain the robust sex differences in prevalence and symptoms of ASD. Past research has demonstrated that males and females are differentially susceptible to chemical agents during early brain development (Geller, Oshiro, Haykal-Coates, Kodavanti & Bushnell, 2001; Schneider et al., 2008). Thus, it is vital to determine if chemical risk factors for ASD interact differentially with sex. Additionally, females are often underrepresented in ASD research and little research has been conducted investigating the effects of PPA in female rats. This study therefore aims to further develop the validity of the PPA animal model of ASD by expanding research to incorporate female and adolescent rats.

Behaviour was assessed using the hole-board apparatus, which has been previously used to measure restrictive, perseverant, repetitive, exploratory and locomotor behaviour (Calamandrei, Pennazza, Ricceri & Valanzano, 1996; File & Wardill, 1975; Meeking, Foley, Tichenoff, Ossenkopp & MacFabe, 2009; Moy et al., 2008). The hole-board apparatus consists of 16 equally spaced wells within an open field chamber allowing measurement of locomotor and nose poke activity of rodents. The hole-board has been successful when measuring

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behaviour using social olfactory cues (Meeking et al., 2009; Moy et al., 2008). For instance, Meeking et al. (2009) used olfactory social cues within the hole-board apparatus to measure perseverant, repetitive and locomotor behaviours in centrally PPA-infused adult male rats. This study found increased perseverant behaviours, repetitive movements, and locomotor activity in PPA-infused rats (Meeking et al., 2009).

The present study used a similar procedure as Meeking et al. (2009) to investigate the effects of intraperitoneal (IP) PPA infusion on locomotor and nose poke activity to social olfactory cues in female adolescent rats. This study additionally examined thigmotaxis variables (i.e. tendency of animal to stay close to the wall) as an indicator of anxiety-like behaviour. Since prior research has reported increased anxiety-like behaviour in systemically PPA-treated females (Foley et al., 2014) and adolescent rats (Shams et al., 2009), it was predicted that intraperitoneally-induced female adolescent rats would show increased anxiety as demonstrated by more time spent in the periphery of the apparatus. Based on previous research with IP injections (Benzaquen et al., 2010), it was hypothesized that IP PPA injections would produce hypoactivity in female rats. If IP PPA alters behaviour in a similar manner as central PPA administration (Meeking et al., 2009), it was hypothesized that PPA-treated female adolescent rats would demonstrate increased repetitive behaviours.

### Methods

#### Subjects

Twelve female Long Evans rats weighing 100-140 g (approximately 30-35 days old) at time of arrival from Charles River Laboratories (Quebec, Canada) were used in the experiment. Animals were kept in facilities for 7 days after arrival from laboratories before testing began. Rats were housed in pairs in standard polypropylene cages (26 cm x 48 cm x 21 cm)

and provided access to water and food (LabDiet RMH 3000) *ad libitum*. Animals were housed in temperature-controlled colony room ( $21 \pm 1$  °C) with lights on from 07:00h to 19:00h. All procedures abided by Canadian Council on Animal Care guidelines and were approved by the University of Western Ontario Animal Use Subcommittee.

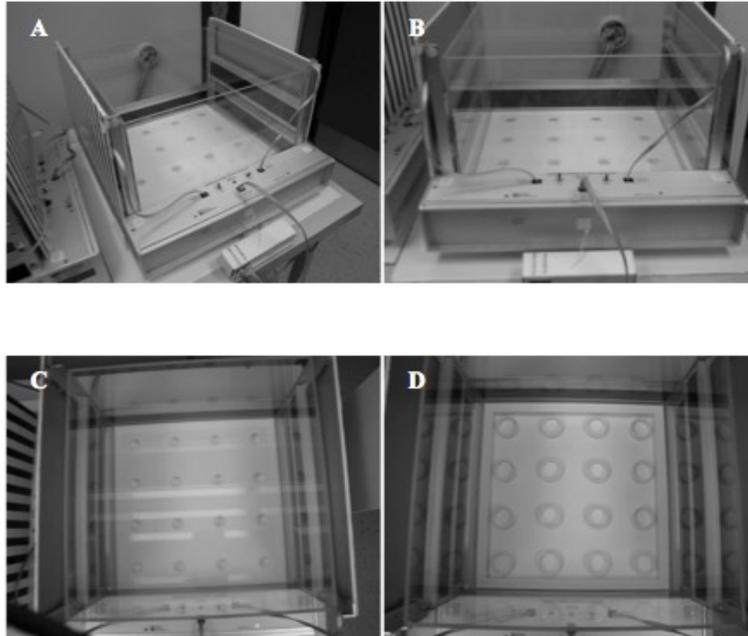
#### Apparatus

Activity was measured in a clear Plexiglas open field chamber (40 cm x 40 cm x 30.5 cm) with a clear plastic lid with air holes and an inserted hole board (see Figure 1). Locomotor activity was monitored using three Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA). To measure thigmotaxis variables, VersaMax software separated the apparatus into distinct centre (30 x 30 cm square) and periphery (7.5 cm wide border) sections. Horizontal movement was recorded using a grid of infrared beams located on all four sides of the chamber (16 beams spaced 2.54 cm apart and 4.5 cm above floor). Vertical movement was recorded using infrared beams on two opposite sides of the chamber (16 beams spaced 2.54 cm apart and 15 cm above floor). Nose poke activity was measured using a hole-board inserted at the base of the chamber. The hole-board consists of 16 equally spaced holes, each 2.54 cm in diameter and 5.08 cm in depth.

#### Drugs

Animals were randomly assigned to receive either PPA (0.26 M, 2 ml/kg,  $n = 6$ ) or Phosphate Buffered Saline (PBS,  $n = 6$ ). PPA was dissolved in 0.1 M saline and injected intraperitoneally at 500 mg/kg. Vehicle was injected at 2 ml/kg. Selected doses were based on results of previous studies (Ossenkopp et al., 2012). All solutions were corrected to pH 7.5. Injections occurred 20 minutes prior to the onset of behavioural testing.

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*Figure 1.* Photographs of hole-board apparatus. (A) side view (B) front view (C) top view with board inserted covering holes (D) top view without board inserted covering holes. Reprinted from “Assessing exploratory and repetitive behaviour in Long-Evans rats in a propionic acid model of autism spectrum disorder,” by M. M. Meeking, K. A. Foley, L. J. Tichenoff, K. P. Ossenkopp, & D. F. MacFabe, 2009, University of Western Ontario, London, Ontario.

### **Experimental Procedure**

There were three phases within the experiment: baseline phase (days 1 and 2), odor exposure phase (days 3 and 4) and test phase (day 5). On all days, locomotor and nose poke activity were recorded within the apparatus for 20 minutes (four 5 minute time bins). As little prior research is available on the timing effects of IP PPA injections, this timeframe was established using prior findings with systemic injections of propionate, which caused peak effects in the brain at about 60 minutes after injection and dissipate soon after (Brusque et al., 1999). Therefore, behaviour in the present study was measured between 20 and 40 minutes after injection. In the baseline phase, rats were handled and habituated to the apparatus. On these days, clean bedding was placed in two separate centre cups (holes 6 and 11) and

baseline nose-poke and locomotor activity was measured. In the exposure and test phase, rats were injected with either PPA or PBS, and placed in apparatus with activity recorded. On these days, one centre hole (either 6 or 11) was filled with clean bedding and one with soiled bedding (either 6 or 11). Each experimental rat kept the same test hole (i.e. soiled bedding hole) throughout all treatment days (either hole 6 or 11, counterbalanced within treatment group). In the exposure phase (days 3 and 4), soiled bedding from an unfamiliar rat cage was used. Soiled bedding was obtained from a different unfamiliar female rat cage for each exposure day. This soiled bedding was used to assess social cues. In the testing phase (day 5), soiled bedding from the experimental rat’s own case was used to replace the unfamiliar soiled bedding. This was used to analyze the presence

of perseverant behaviours, by assessing if the rat would appropriately respond the change in social stimuli. Locomotor and repetitive body movements were measured throughout testing procedure. Rats were weighed daily to monitor health.

### **Behavioural Measures**

Locomotor activity was analyzed using 8 individual measures. Horizontal activity measures included: total horizontal distance travelled (cm), horizontal movement time (s), and number of horizontal movements separated by a 1 s stop time. Vertical activity measures included: total vertical movement time (s), and number of vertical movements separated by a 1 s stop time. Repetitive locomotor activities were measured using number of revolutions (number of movements in clockwise or counterclockwise circle of minimum 2 inches in diameter), and number of stereotypic movements (repeated breaking of the same infrared beam separated by at least 1 s).

Nose poke behaviour was analyzed using 3 individual measures: total nose pokes (total number of nose pokes on all holes across entire testing session), hole category (number of nose pokes as classified by hole location in apparatus), and difference scores (demonstrate preferences for empty, clean bedding or soiled bedding hole). Hole categories were split into three sections based on location within apparatus: corner holes (holes 1, 4, 13 and 16), centre holes (holes 6, 7, 10 and 11) and wall holes (holes 2, 3, 5, 8, 9, 12, 14 and 15) (see Figure 2). Difference scores were calculated by examining the difference in number of nose poke for empty, clean or soiled bedding holes. Total nose pokes per hole [empty hole (E), clean bedding hole (CB) and soiled bedding hole (SB)] were calculated as a percentage of total nose pokes per testing session to control for individual differences in nose poke behaviour. Preference for soiled as opposed to clean

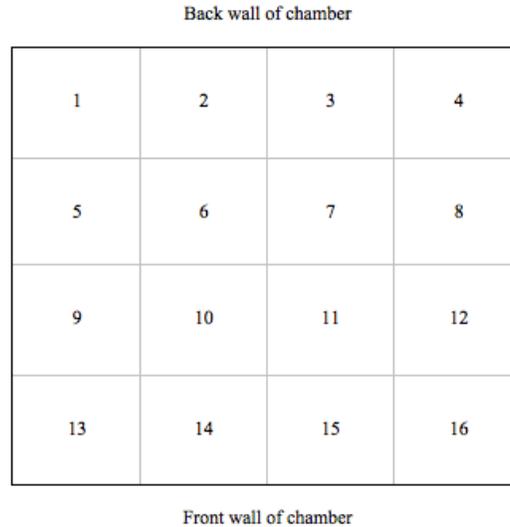
bedding was calculated by subtracting the CB percent from the SB percent, with positive scores indicating a preference for soiled bedding and negative scores indicating a preference for clean bedding. Preference for soiled bedding as opposed to an empty hole was calculated by subtracting the E percent from the SB percent, with positive scores indicating a preference for soiled bedding and negative scores indicating a preference for an empty hole.

Thigmotaxis variables were analyzed using 6 different variables in both the periphery and the centre. Variables included total duration (s), total distance travelled (cm), horizontal movement time (s), number of horizontal movements, vertical movement time (s), number of vertical movements and number of stereotypic movements in both the centre and periphery of the apparatus. Locomotor activity variables were time corrected (divided by duration in centre or periphery).

### **Statistical Analysis**

All statistical analyses were performed using IBM Statistics 21 (formerly Statistical Package for the Social Sciences, SPSS). Separate statistical analyses were conducted for each behavioural variable. A one-way analysis of variance (ANOVA) was conducted on the second day baseline data to control for pre-existing group differences prior to drug treatment. If baseline ANOVA was non-significant, a repeated measures split-plot ANOVA was used to analyze main and interaction effects during exposure days. The between-subjects factor was drug treatment (2 levels: PPA and PBS) and within-subjects factor was treatment day (2 levels: day 3 and 4). For any variable in which the baseline ANOVA was significant, a repeated measures analysis of covariance (ANCOVA) was performed, using the mean total of baseline data as a co-variate to control for pre-existing group differences. Where appropriate (i.e., if there was a significant

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*Figure 2.* Layout of holes within hole-board apparatus showing corner holes (1, 4, 13, 16), centre holes (6, 7, 10, 11) and wall holes (2, 3, 5, 8, 9, 12, 14, 15). Reprinted from “Assessing exploratory and repetitive behaviour in Long-Evans rats in a propionic acid model of autism spectrum disorder,” by M. M. Meeking, K. A. Foley, L. J. Tichenoff, K. P. Ossenkopp, & D. F. MacFabe, 2009, University of Western Ontario, London, Ontario.

day x drug interaction), one-way ANOVAs were conducted on data from each day individually to determine the effect of drug treatment on individual days. For the test phase (day 5), a one-way ANOVA was conducted if the baseline ANOVA was non-significant and a one-way ANCOVA was used if baseline ANOVA was significant. Significance was set to  $\alpha = .05$ .

### Results

#### Body weight

**Baseline.** A one-way ANOVA was performed on second day baseline weights and was non-significant. Therefore, there were no pre-existing differences in the weights of treatment groups.

**Treatment Days.** A repeated-measures ANOVA revealed a significant main effect for day,  $F(2, 20) = 152.36, p < .001$ , with weight increasing throughout treatment days (see Figure 3). However, there was no significant main effect of drug or day x drug interaction effect. Thus, PPA- and PBS- treatment groups gained

comparable levels of weight throughout treatment days.

#### Locomotor Variables

**Baseline.** One-way ANOVAs were performed on second day baseline data for each locomotor variable. The one-way ANOVAs were non-significant for total distance travelled, number of horizontal movements, horizontal movement time, number of vertical movements, vertical movement time, number of stereotypic movements and number of revolutions. Therefore, there were no significant pre-existing differences between groups prior to the onset of treatment.

**Horizontal activity measures.** A repeated-measures ANOVA revealed a significant main effect of day,  $F(2,20) = 4.07, p < .05$  for total number of horizontal movements, with a decrease in number of horizontal movements across treatment days. For total number of horizontal movements there was a non-significant main effect of drug and non-

## PPA AND ASD BEHAVIOUR IN FEMALE RATS

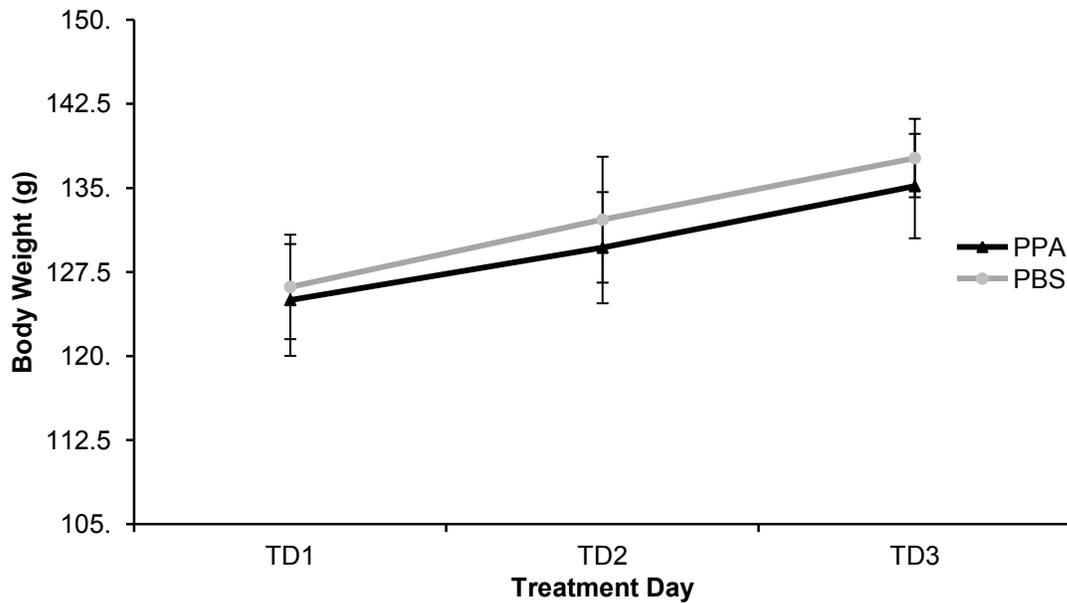


Figure 3. Mean body weights  $\pm$  SEM across treatment days in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals. Significant main effect of day was found with animals increasing in body weight across treatment days. No significant main effect of drug or interaction effect was found.

significant interaction effect (see Figure 4).

Therefore, both groups significantly decreased in number of horizontal movements across treatment days. No significant main effect of day, main effect of drug, or interaction effect were found for total distance travelled or total horizontal movement time (see Figure 5).

**Vertical activity measures.** Analyses revealed no significant main effect of day, main effect of drug, or interaction effect for total number of vertical movements or vertical movement time (see Figure 6). Thus, PPA and PBS- treated animals showed comparable levels in all vertical activity measures.

**Repetitive activity measures.** Analyses for number of stereotypic movements and total number of revolutions showed a no significant main effect of day, main effect of drug, day x drug interaction effect (see Figure 7). Therefore, PPA animals showed comparative repetitive behaviour to PBS controls on all repetitive activity measures.

### Nose poke Variables

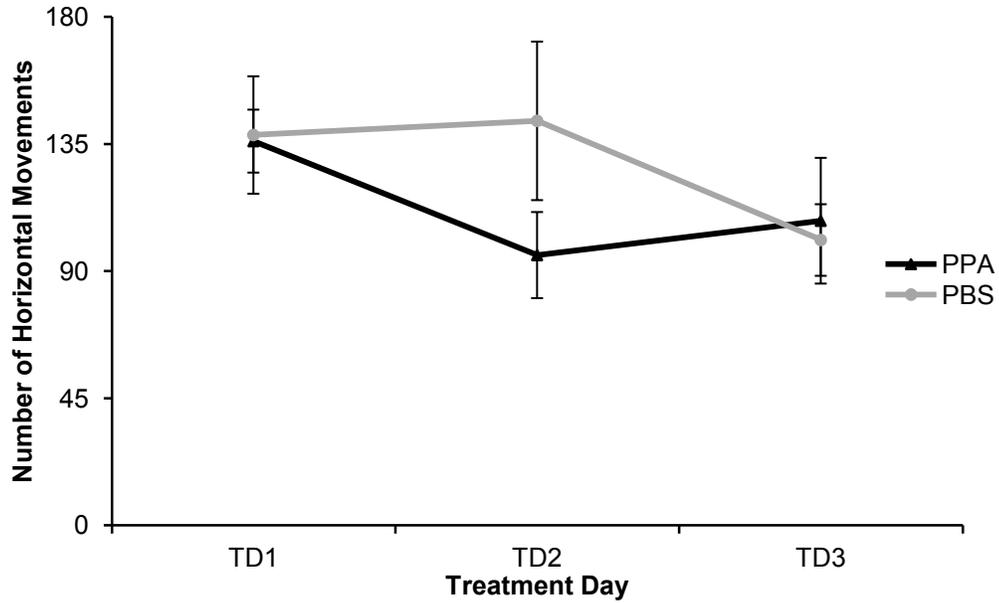
Due to an error with the hole-board apparatus, the computer recorded no nose-poke data. Whether this is due to a technical error or the small size of the adolescent rats used is yet to be determined.

### Thigmotaxis Variables

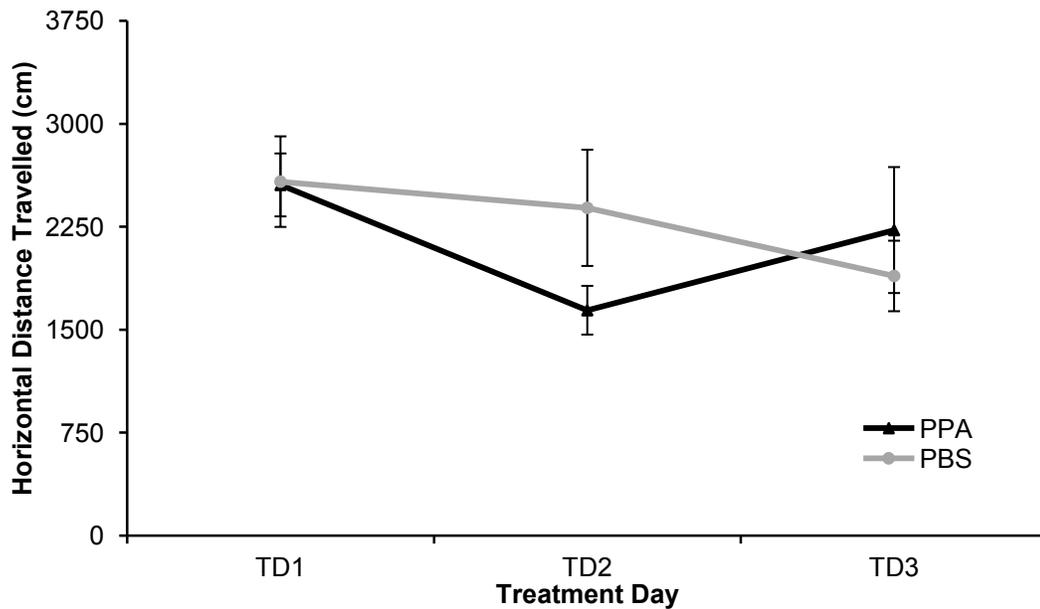
Locomotor measures were corrected for time spent in the centre or periphery of apparatus by dividing each locomotor variables by total duration in the respective area. Corrected values were used for all analyses and graphs.

**Periphery Variables.** Baseline ANOVAs for peripheral duration, total peripheral distance travelled, horizontal movement time, number of vertical movement, vertical movement time, and stereotypic movements were found to be non-significant. For peripheral duration, there was a significant main effect of day,  $F(2,20) = 5.73$ ,  $p < .01$  but no significant main effect of drug, or day x drug interaction effect, (see Figure 8).

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*Figure 4.* Mean number of horizontal movements separated by  $1 s \pm SEM$  in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. Significant main effect of day was found with animals decreasing in numbers of horizontal movement across days. No significant main effect of drug or interaction effect was found.



*Figure 5.* Mean horizontal distance travelled  $\pm SEM$  in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. No significant main or interaction effects were found.

## PPA AND ASD BEHAVIOUR IN FEMALE RATS

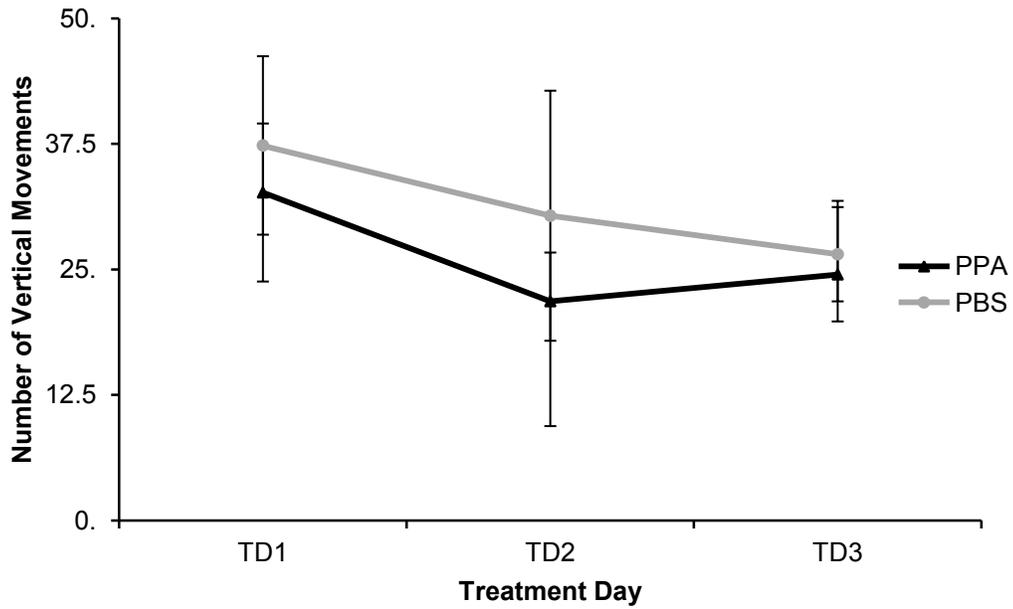


Figure 6. Mean number of vertical movements separated by  $1 \text{ s} \pm \text{SEM}$  in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. No significant main or interaction effects were found.

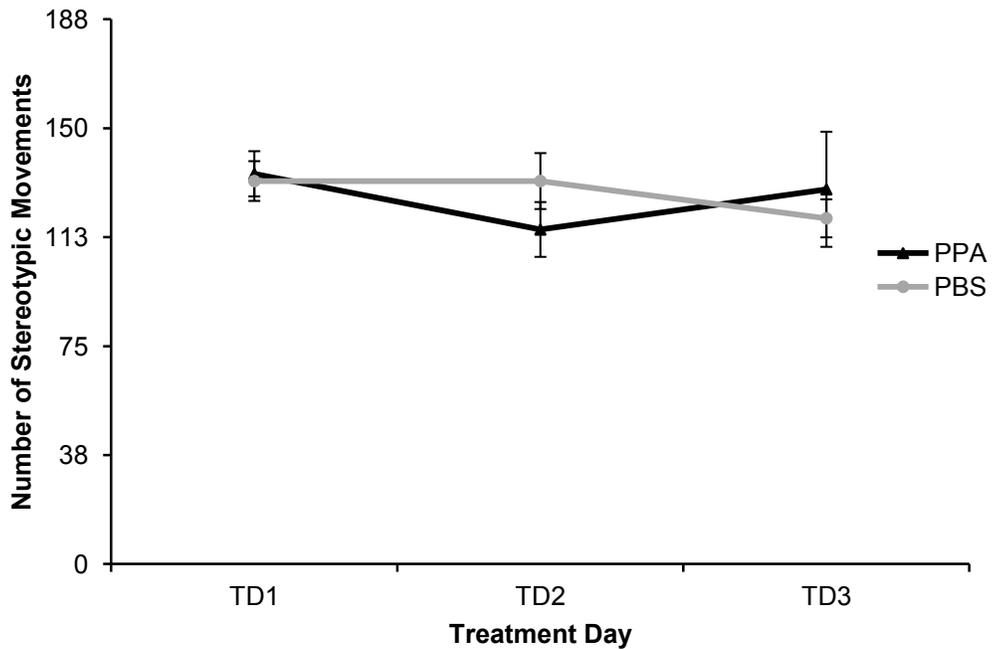


Figure 7. Mean number of stereotypic movements  $\pm \text{SEM}$  in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. No significant main or interaction effects were found.

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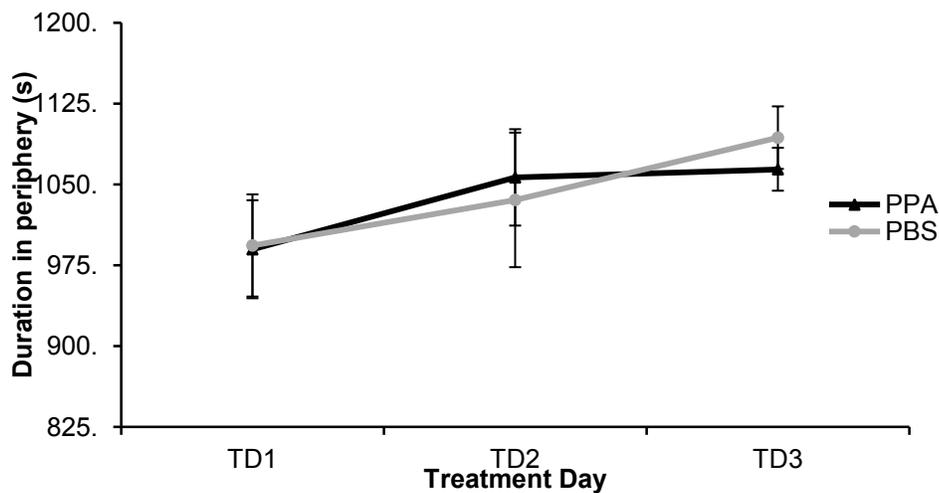


Figure 8. Mean duration in periphery  $\pm$  SEM in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. A significant main effect of day was found with peripheral duration increasing across treatment days. No significant main effect of drug or interaction effect was found.

Thus, both groups showed significantly increases in time spent in periphery across days.

Further analyses revealed no significant main effects of day on horizontal distance, horizontal movement time, number of vertical movements, vertical movement time, and stereotypic movements in the periphery (see Figure 9). Further, there was no significant effect of drug on horizontal distance travelled, horizontal movement time, number of vertical movements, vertical movement time, and stereotypic movements. Additionally, no significant day  $\times$  drug interaction effects were found using periphery data for horizontal distance, horizontal movement time, number of vertical movements, vertical movement time, and stereotypic movements. Therefore, both PPA- and PBS- infused animals showed no significant changes in peripheral locomotor variables across treatment days.

**Centre Variables.** Baseline ANOVAs were non-significant for duration, distance travelled, horizontal movement time, number of vertical

movements, vertical movement time, and stereotypic movements in the centre of the apparatus. Analyses found a significant main effect of day on centre duration,  $F(2,20) = 5.73$ ,  $p < .01$  with no significant main effect of drug, or interaction effect (see Figure 10). Thus, both groups significantly decreased in time spent in the centre of the apparatus across treatment days. Further analyses revealed no significant main effect of day, main effect of drug or interaction effect for total distance travelled, horizontal movement time, number of vertical movements, total vertical movement time, and stereotypic behaviours, within the centre of the apparatus (see Figure 11). Thus, PPA and PBS animals showed no changes in locomotor behaviour across treatment days within the centre of the apparatus.

### Discussion

Based on previous research, this study hypothesized that intraperitoneal PPA administered to female adolescent rats would produce hypoactivity, increased repetitive

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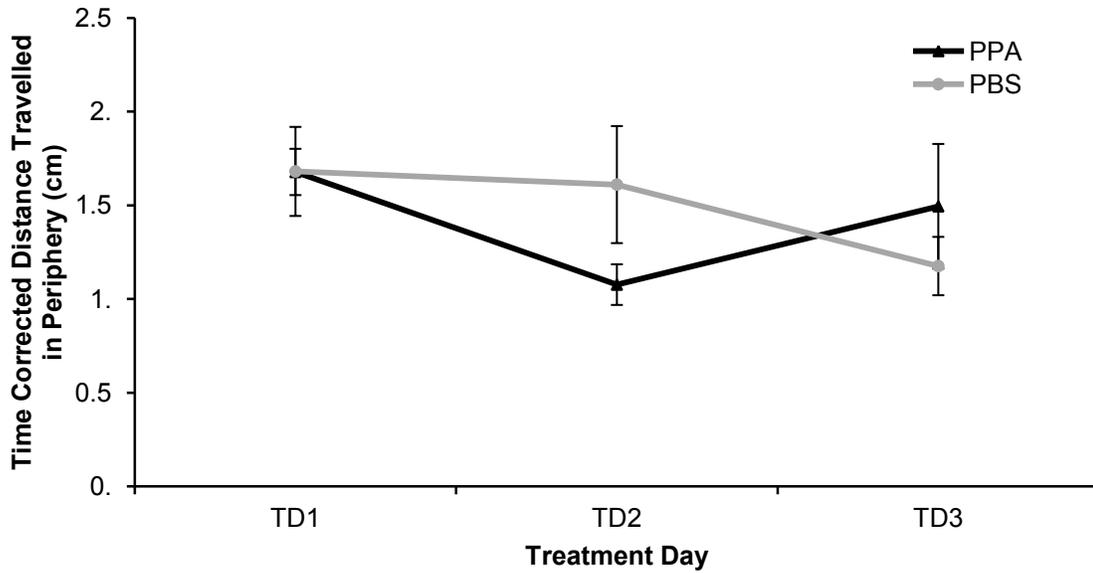


Figure 9. Mean time corrected distance travelled in periphery  $\pm$  SEM in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. No significant main or interaction effects were found.

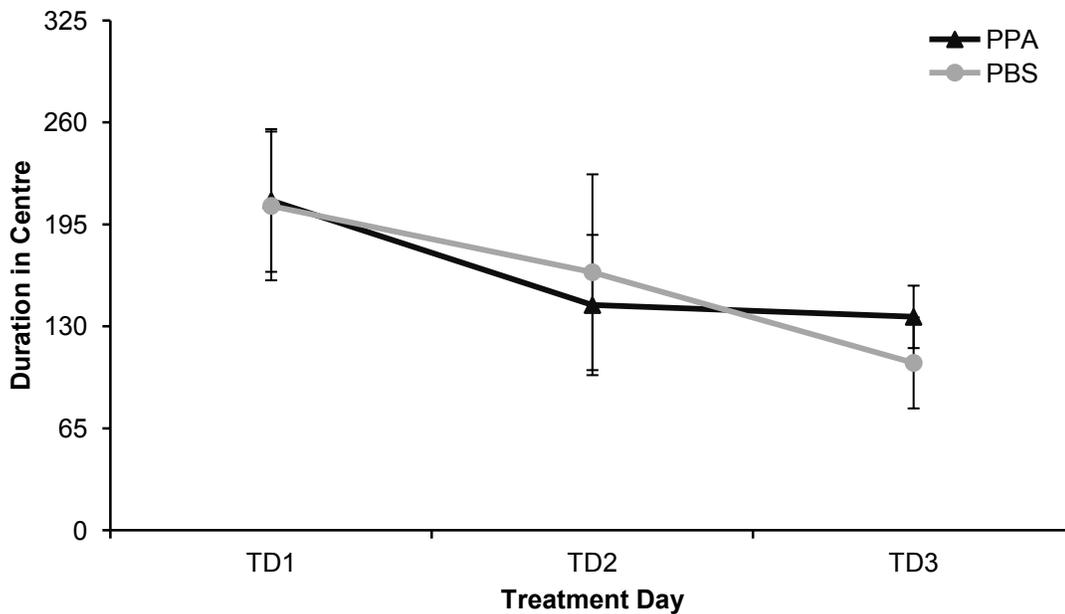
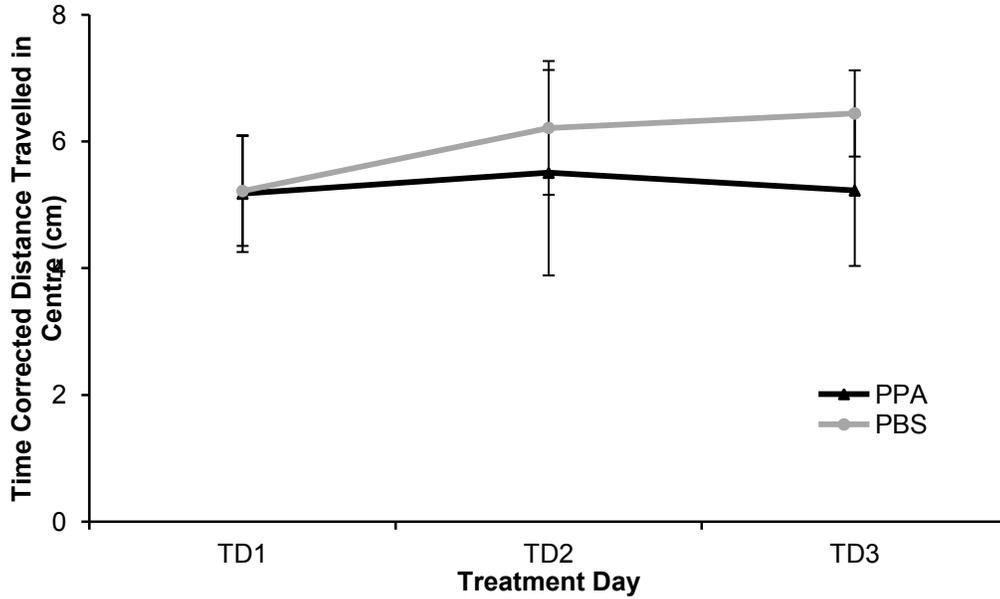


Figure 10. Mean duration in periphery  $\pm$  SEM in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. A significant main effect of day was found with centre duration decreasing across treatment days. No significant main effect of drug or interaction effect was found.

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*Figure 11.* Mean time corrected distance travelled in centre  $\pm$  SEM in PBS ( $n = 6$ ) and PPA (500 mg/kg,  $n = 6$ ) injected animals across treatment days. No significant main or interaction effects were found.

behaviours, and greater anxiety-related behaviours as measured by thigmotaxis (i.e. time spent in periphery). Contrary to these expectations, PPA-treated animals did not significantly differ from PBS controls for any locomotor, stereotypic or thigmotaxis variables. Therefore, the results of the present study indicate that IP injections of PPA did not significantly alter locomotor, repetitive or anxiety-related behaviours in female adolescent rats. Additionally, all animals, regardless of treatment, showed a significant decrease in duration in the centre and a significant increase in duration in the periphery of the apparatus across days.

In the current study, both PPA- and PBS-treated animals showed a similar decrease in number of horizontal movements across treatment days. This suggests habituation to the apparatus, as characterized by decreased exploratory or locomotor behaviour over repeated exposures to the novel environment (Leussis & Bolivar, 2006). Consistent with this

habituation effect, animals showed decreasing exploratory behaviour, demonstrated by a decrease in centre duration and increase in periphery duration across days. It is unlikely that this change in behaviour in both PPA and PBS-treated rats was due to an increase in anxiety-like states as there was comprehensive handling and baseline procedure conducted prior to treatment days. Over the course of the experiment, it is likely that animals continued to habituate to procedures and apparatus.

The above explanation is also influenced by the fact that there was soiled bedding in centre holes to serve as social olfactory cues. Centre duration may therefore also be used to assess social abilities of animals. Socially salient olfactory cues (i.e., soiled bedding from an unfamiliar rat cage) were placed in a centre hole on the first two treatment days and removed on the last day. Rats may be attracted to novel social cues during the first treatment day, but habituate to the stimulus novelty over days leading to less attraction to the stimulus, and

thus a decrease in time spent in the centre of the apparatus. Although there was bedding from different rats in the holes on treatment days 1 and 2, the salience or novelty of the odor may not have been sufficient to attract attention on day 2.

On the last day, when social olfactory cues were no longer present, animals may show lowest levels of attraction to the centre. Although this may be due to habituation, it may also demonstrate species-appropriate attraction to socially salient cues. On earlier days, when social cues were present, animals showed higher levels of time in centre than on the last day when social cues were no longer present. This is consistent with previous research that found systemic PPA treatments to impair social behaviours in males but not in females (Benzaquen et al., 2010). As previous research has consistently found impaired social behaviours in PPA-treated males (Shultz et al., 2008; Benzaquen et al., 2010), future research should examine potential sex-specific effects of PPA on multiple social behaviours as an explanation for sex differences in social symptoms of ASD.

Intraperitoneal PPA in adolescent females did not have significant physiological or behavioural effects. Physiological effects were assessed using body weight, with both treatment groups showing significant increases in body weight across treatment days. This is inconsistent with previous reports that systemic PPA may cause short-term reductions in normal weight gain. For instance, Foley et al. (2014) found that postnatal PPA-treated female pups were significantly lighter than controls in early life, yet this weight difference was non-significant by adolescence. This decreased weight gain may be due to PPA-induced aversive internal cues, enhanced satiety or reduced gastric emptying, which can suppress food intake and limit immediate weight gain in PPA-treated animals (Darwiche et al., 2001;

Liljeberg & Bjorck, 1996; Ossenkopp et al., 2012). Accordingly, numerous studies have found that PPA may cause aversive internal states in rats (Ossenkopp et al., 2012), and reduce food intake or enhance feelings of satiety in ruminants (Arora, Sharma, & Frost, 2011) and humans (Liljeberg, Lonner & Bjorck, 1995; Ruijschop, Boelrijk, & te Giffel, 2008). In the current study, PPA treated animals showed a trend towards lower weight gain than controls, although this trend was non-significant. The inconsistencies found may be a result of the different developmental timepoints of injections and/or the number of injections used in the studies. This suggests that IP PPA treatment of one injection on three consecutive days may not have the same aversive internal cues or physiological reactions as in previous studies, such as Foley et al. (2014), which administered multiple injections per day and tested behaviour over longer time periods.

The present findings also indicate no significant effects of PPA on locomotor and repetitive behaviours. Mixed results have been found regarding PPA's effect on locomotor activity in rats, with some studies reporting hypoactivity (Benzaquen et al., 2010; Ossenkopp et al., 2012; Shams et al., 2009), hyperactivity (MacFabe et al., 2007; MacFabe et al., 2008; Thomas et al., 2010) or no changes in activity (Foley et al., 2014; MacFabe et al., 2011; Shultz et al., 2008) in PPA-injected animals. Specifically, studies examining acute systemic and IP PPA-infusions in male and female adolescent rats reported hypoactivity (Benzaquen et al., 2010; Shams et al., 2009), with prenatal and postnatal PPA producing no changes in locomotor behaviour (Foley et al., 2014). Additionally, where previous studies consistently report PPA to increase repetitive behaviours in adolescent and adult rats (Foley et al., 2014; MacFabe et al., 2007; Meeking et al., 2009), this experiment did not find any

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significant PPA-induced differences in repetitive behaviours.

One potential reason for the non-significant results found may be that PPA did not reach high enough concentrations within the brain to cause behavioural changes. Previous research has found that PPA impacts numerous neural mechanisms, which in turn cause changes in repetitive, locomotor and exploratory behaviours. PPA may influence areas of the brain related to locomotor activity via its effects on the inhibition of the Na<sup>+</sup>/K<sup>+</sup> ATPase (Wyse et al., 1998), rise in intracellular calcium release (Nakao et al., 1992), elevated nitric oxide levels (Nicot, Otto, Brabet & Diccico-Bloom, 2004) and enhanced NMDA receptor sensitivity (de Mattos-Dutra et al., 2000). PPA also causes intracellular acidification, which has been linked to increased locomotor activity (Brouillet, Jacquard, Bizat & Blum, 2005), and may result in increased synthesis and release of several neurotransmitters related to locomotor activity, including dopamine, glutamate, serotonin and norepinephrine (Cannizzaro et al., 2003; Remblier, Pontcharraud, Tallineau, Piriou & Huguet, 1999; Severson et al., 2003). These increased neurotransmitter levels in the brain, specifically serotonin and dopamine, have been linked to repetitive and stereotyped movement in rodents (Langen, Kas, Staal, van Engeland & Durston, 2011). Therefore, PPA may influence locomotor and repetitive behaviours by altering neural function. However, in the present study, PPA levels may not have reached adequate levels within the brain to cause changes in overt behaviour. One key difference between this study and prior research is that much previous work has used intraventricular (MacFabe et al., 2007, 2008), intracerebroventricular (Shultz et al., 2008, 2009), central (Meeking et al., 2009) or systemic (Foley et al., 2014; Ossenkopp et al., 2012; Shams et al., 2009) forms of injection. Current findings may therefore demonstrate that IP injection circulates PPA differently than other

forms of injections, and potentially may not lead to the levels of neural PPA necessary to cause substantial behavioural and physiological effects as seen in previous studies.

An alternative reason for the non-significant effects of PPA in the current study may be inherit sex differences in PPA's influence on behaviour. Where numerous past studies on male adolescents have found systemic PPA-induced effects on locomotor and repetitive behaviours (Benzaquen et al., 2010; Shams et al., 2009), the current study found no significant PPA-induced effects in female adolescents. Further, Gottlieb (2014) conducted a study on male adolescent rats in conjunction with the present experiment using the same experimental procedure, dosages, and timing. Contrary to present findings, this study found numerous significant IP PPA-induced effects on males, such as hypoactivity. Therefore, the treatment method used in the present study allowed for substantial effects of PPA in male, but not female, adolescent rats. This shows the IP PPA injections can have significant effects on the brain and behaviour, which was seen in adolescent males but not females. This is consistent with previous findings that report sex differences in the PPA animal model such as social impairments shown in male, but not female, rats (Benzaquen et al., 2010).

Prior research may suggest that females are differentially susceptible to the effects of PPA on the brain. Brain profiles in ASD autopsies and centrally-infused PPA research have consistently reported innate neuroinflammation, including activated microglia and reactive astrogliosis, and oxidative stress (MacFabe et al., 2007, MacFabe, Cain, Boon, Ossenkopp & Cain, 2011; Vargas, Nascimbene, Krishnan, Zimmerman & Pardo, 2005). These effects within the brain may occur in a sex-specific manner. For instance, past research has found sex differences in the colonization and function of microglia during normal brain development,

thereby leading to different windows of vulnerability in males and females (Schwarz & Bilbo, 2012). Further, estrogen has been shown to have numerous neuroprotective effects, such as the ability to protect against neuronal cell death caused by oxidative stress or influence astrocyte activation (Cordeau, Lalancette-Hebert, Weng & Kriz, 2008; Green & Simpkins, 2000; Sobocanec et al., 2008; Tomomi et al., 2002). In animal models, estrogen has been shown to slow neuronal cell death following traumatic brain injury, cerebral schema and Parkinson's disease (Green & Simpkins, 2000). Thus, estrogen may have protected the brain from oxidative stress cause by PPA-infusion within the present study. Males and females may also show differences in gut microbiota abnormalities associated with ASD, as demonstrated by research in the VPA animal model of ASD (de Theije et al., 2014). Overall, these findings show that PPA may have different neurological and physiological effects in females and males.

PPA's sex-specific influence on behaviour may account for sex differences seen in human ASD symptoms. For instance, the current study found no effect on locomotor or repetitive behaviours in female adolescent rats. These findings are consistent with sex differences seen in ASD in humans, where females show lower levels of externalizing behaviours (e.g., repetitive, restricted or stereotyped behaviours) or hyperactivity relative to males (Hartley & Sikora, 2009; Mandy et al., 2012; Werling & Geschwind, 2013). Additionally, findings implied intact social skills in females, which is consistent with human sex differences finding lower social disturbances in ASD females. Specifically, young females with ASD may show less overt social deficits as they are often less disruptive, better able to mimic other's behaviours, and more motivated to be socially interactive (Holtman et al., 2007). Therefore, sex-specific effects of PPA may be a potential

explanation for sex differences seen in human ASD symptoms. Further research must be conducted to examine which specific ASD symptoms are influenced by PPA and the sex-specific manner in which these effects might occur.

In addition to differences in symptoms, sex-specific metabolism and effects of PPA may provide a potential epigenetic explanation for sex differences in ASD prevalence. Sex differences in ASD prevalence may be due to increased protective effects against ASD impairments in females (Robinson, Lichenstein, Anckarsäter, Happé & Ronald, 2013). The female protective effect hypothesizes that females may have greater resilience to ASD and therefore require greater familial etiologic load to manifest ASD impairments. Further, recent research on chromosomal structural variations in ASD has found that rare genetic de novo insults are larger and more disruptive in females than in males (Gilman et al., 2011; Levy et al., 2011). In the current study, effects of PPA may be defended against by inherit protective factors in females, such as estrogen. Further investigation into the effects of female specific protective agents against PPA should be examined to validate this hypothesis.

### **Future Research**

The current study was a preliminary investigation into the effects of IP PPA on the behaviours of female adolescent rats. Due to the small sample size used in the current study, future research is needed to further confirm and clarify present findings on the sex-specific effects of PPA. Discrepancies between previous and current results may be attributed to differences in procedure, such as drug dosage, method of administration, time elapsed since injection and number of injections/infusions. For instance, prior research, which found significant results using systemic PPA injections, had injected animals twice daily (Benzaquen et al.,

2010; Foley et al., 2014) or had examined behaviour for a greater number of testing days (Benzaquen et al., 2010; Foley et al., 2014; Shams et al., 2009). In addition, the length of exposure to PPA may impact effects on behaviour. For example, Meeking et al. (2009) found that a low dose of centrally-infused PPA caused similar effects as a high dose of PPA on locomotor measures, however not until later in the infusion schedule. Thus, numerous procedural variations may account for differences found between past and current findings and future research should look to clarify the varying results found due to procedural differences.

In addition to this, different developmental time periods may elicit different behaviours in females in response to PPA. For instance, prenatal PPA exposure produced various significant effects on behaviour not seen with postnatal injections, such as increased number of vertical movements and decreased time spent in the centre of the apparatus (Foley et al., 2014). Further, prenatal PPA exposure may have different effects if re-exposed to PPA postnatally, such as increased anxiety on elevated plus maze in females (Foley et al., 2014). Further research should examine the specific effects of PPA within different developmental periods and the potential interactions between prenatal PPA exposure and later environmental influences. This may allow for better understanding of the epigenetic effects of PPA throughout the developmental trajectory. Overall, future research should examine the sex-specific effects of PPA on physiology and behaviour within distinct developmental periods as a potential explanation for sex differences in human ASD symptoms and prevalence.

### Conclusions

The present study found that PPA did not have significant behavioural or physiological impacts on female adolescent rats. As significant

impacts were found in male adolescent rats in a study performed simultaneously to the present one (Gottlieb, 2014), findings implicate potential sex-differences in the effects of PPA. As PPA has been correlated with ASD symptoms in numerous ways, sex-differences in the response to PPA may account for some of the sex-differences seen in the prevalence and demonstration of ASD within the population. Further research is needed to clarify the sex-specific effects of PPA, and define its relation to ASD in humans.

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