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The Effect of Propionic Acid on the Habituation to Social and Non-social Odour Cues in Adult Male Rats

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Abstract

The enteric bacterial metabolite, propionic acid (PPA), elicits physiological and behavioural changes in rodents reminiscent of autism spectrum disorder (ASD). This includes abnormal sensory processing and social behaviour. ASD may contribute to social deficits through impaired habituation; therefore, the present study examined the effects of intraperitoneal PPA on the habituation to social and non-social odours. Adult male rats were injected daily with PPA or the vehicle control, and for 3 days, habituated to a conspecific odour or vanilla extract for 10 minutes. On day 4, rats were exposed to a novel conspecific odour or almond extract for 10 minutes to observe dishabituation. Behaviours were measured in the open-field and analyzed via an automated system and by the manual scoring of video-recordings. Results from both scoring methods strongly correlated with one another. PPA treatment significantly increased repetitive behaviours and hypoactivity. Drug and odour had no significant effects on odour habituation, although PPA non-social rats displayed reduced habituation for entries into the odour quadrant and sniffing. Group differences were insignificant. No dishabituation to the odour was observed in all groups. However, an insignificant, subtle reduction in dishabituation to the open-field was seen in PPA groups for total distance travelled. PPA may influence odour discrimination in rodents and contribute to sensory habituation deficits in ASD. Differences in body temperature and weight post-injection were measured to monitor a potential sickness response from the bacterial by-product. Results for PPA rats did not differ from controls, suggesting that PPA exerts its effects through other mechanisms.

Keywords

Keywords: autism spectrum disorder; propionic acid; bacterial metabolite; short chain fatty acid; habituation; dishabituation; hypoactivity; repetitive movements; social and non-social odours; rat model

Summary for Lay Audience

Disturbances involving enteric metabolites highlight the gut-brain axis, as they may play a role in the etiology of autism spectrum disorders (ASDs). The gut bacterial by-product, propionic acid (PPA), is of interest as an etiological factor in ASD, as it can cross the blood-brain barrier and affect central nervous system functioning. PPA can elicit ASD-like symptoms in rodents including abnormal social behaviour, repetitive movements, sensory processing deficits, and hypoactivity. ASD may contribute to social deficits through impaired habituation to sensory and social stimuli, therefore, the present study examined the effects of systemic PPA injections in adult male rats on habituation to social and non-social odors. Habituation is expressed when there is a diminished response to a stimulus after repeated exposure. For rats with properly functioning olfactory systems, habituation occurs when an odour stimulus is sniffed less often over time. Dishabituation occurs when a novel odour is introduced, and an increase in odour investigation is observed.

Rats were injected daily with PPA or the control, phosphate buffered saline. Behaviours were detected in the open-field via an automated system and by video-recordings. Results from both scoring methods strongly correlated with one another. Rats habituated to same social or non-social odour for 10 minutes for 3 consecutive days. On the 4th day, rats were exposed to a different social or non-social odour to observe dishabituation.

PPA was effective, as treatment groups displayed significantly more hypoactivity and repetitive behaviours compared to controls. Drug and odour had no significant effects on odour habituation, however PPA non-social rats habituated the least. Group differences were insignificant. No groups showed significant dishabituation to the novel odours, although only PPA treated rats showed a reduction in odour investigation. Group differences were also insignificant. Results suggest that PPA may have a general effect on odour discrimination in rodents, and that it may contribute to social deficits in ASD by impairing habituation to sensory cues.

Body temperature and weight were monitored throughout the experiment to see if the bacterial product induced sickness. Results from PPA treated rats did not differ from controls, suggesting PPA exerts its effects through other mechanisms.

Co-Authorship Statement

All experiments were performed by Cashmeira-Dove Tyson. Drs. Klaus-Peter Ossenkopp, and Martin Kavaliers contributed to the experimental design and the editing of the manuscripts, along with PhD candidate Indra Bishnoi.

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1.0 Introduction

1.1 Autism Spectrum disorder

Autism spectrum disorders (ASD) are a set of neurodevelopmental alterations that affect how individuals socialize, communicate, and behave. The hallmark symptoms of ASD include repetitive and abnormal movements, stereotypic behaviours, cognitive delay, and impaired language and social skills (Arndt et al. 2005). ASD affects 1 in every 44 children and is over 4 times more likely to be diagnosed in males than females (Centre for Disease Control and Prevention, 2018). Common comorbidities among those with ASD include anxiety disorders, and gastrointestinal issues (Skokauskas & Gallagher 2010; Horvath et al., 1999). According to family and twin studies, monozygotic twin concordance rates for ASD are significantly higher than dizygotic twins, therefore ASD is considered the most heritable neurodevelopmental disorder (Bailey et al., 1995; Hallmayer et al., 2011). However, through quantitative analysis and structured diagnostic assessment of a large sample of ASD patients, Hallmayer and colleagues' (2011) California Autism Twins Study showed that shared twin environment and genetic heritability show similar rates of susceptibility to develop ASD, and that heritability concordance rates may be overestimated due to shared environments. There is also a notable percentage of discordant monozygotic twins for ASD (Hallmayer et al., 2011), and when both twins have the disorder, there is heterogeneity in symptoms and severity (Hu et al., 2006), suggesting that non-genetic factors play a role in the etiology.

1.2 The gut-brain axis

A variety of environmental factors can affect central functioning, and more recently, ASD research has been focusing on factors that alter the composition of the gut microbiome and their subsequent effects on behaviour. The central nervous system and the enteric nervous system share an important reciprocal connection termed the gut-brain axis. These systems communicate bi-directionally via the neural system, neurotransmitters, hormones, gut metabolites, and various components involved in the immune response (Collins, 2011). The mammalian gut hosts over 500 different species of bacteria, all of which modulate a variety of homeostatic functions both within and outside of the gastrointestinal (GI) tract, including metabolic, physiological, and immunological responses (Collins, 2011). The bacterial composition of the GI tract is heavily influenced by a variety of factors such as host physiology and immune status, along with environmental factors such as stress, diet, pathogens, and drug usage, especially antibiotics (Collins, 2011; Al-Orf et al., 2018). When there is a disruption of the microbiota, dysbiosis occurs, which has been implicated in GI, metabolic, and psychiatric disorders (Collins, 2011; Cryan et al., 2020).

1.2.1 Gastrointestinal issues, anxiety, and ASD

The relationship between intestinal bacteria and the brain is evident from studies with germ-free animals. For example, Kelly et al. (2016) performed a fecal transplant where microbiota from depressed psychiatric human patients was transplanted into microbiota-depleted rats. The results showed significant physiological and behavioural changes in the rats including an increased

proinflammatory cytokine profile, greater cortisol output, as well as increased anxiety-like and anhedonia-like behaviour (Kelly et al., 2016). People with depression display a different gut microbial profile than people without depression, as there is a reduction in diversity and healthy bacteria (Liang et al., 2018), which may contribute to some behavioural symptoms. Mood disorders and ASD alike both show that anxiety can influence the microbiome, or vice versa. About 40-55% of youth with ASD experience anxiety disorders (Van Steensel et al., 2011; White et al., 2009), however anxiety is more prevalent in those with ASD and gastrointestinal issues (Mazurek et al., 2013). Gastrointestinal issues were considered if they were ongoing for over 3 months and included at least one of the following symptoms: constipation, abdominal pain, bloating, diarrhea, and/or nausea (Mazurek et al., 2013). It is also more common for neurotypical people with gastrointestinal issues to have anxiety disorders (Lee et al., 2009; Hartono et al., 2012). The DSM 5 defines general anxiety disorder, the most common anxiety disorder, as excessive apprehensive expectation about various events or activities occurring more days than not for at least 6 months (American Psychiatric Association, 2013).

Similar to depression, current ASD research indicates that the microbiome of those with ASD differs from their neurotypical peers (Grimaldi et al,, 2018; Sharon et al., 2019; Cryan et al., 2020). Representative research of this includes a study where gut microbiota from humans with ASD was transplanted into germ-free mice. It was found that the mice developed ASD-like behaviours such as

increased repetitive behaviours, decreased locomotor activity, and decreased communication via ultrasonic vocalization (Sharon et al., 2019).

Additional to ASD and gastrointestinal issues, literature shows that there is a link between experiencing anxiety and behavioural symptoms of ASD, specifically repetitive behaviours, and social abnormalities. Symptoms are reported more severe when anxiety is greater (Settipani et al., 2012; Van Steensel et al., 2012; Rogers et al., 2012).

1.2.2 The short chain fatty acid, propionic acid

There is accumulating evidence for associations between disturbances of the GI tract and symptoms of ASD in humans and rodents (MacFabe, 2007, MacFabe, 2008, Foley et al., 2014, Lobzhanidze, 2018; Khang et al., 2017; 2019). Of particular interest are short-chain fatty acids (SCFAs) that are both products of specific categories of bacteria as well as undigested, or partially digested, by-products of fermentation of dietary carbohydrates and fibers (van der Hee & Wells, 2021). Current research places large emphasis on the SCFA, propionate, also called propionic acid (PPA) (Meeking *et al*., 2020). PPA is mainly produced by *Clostridium* and *Bacteroides* bacteria (van der Hee & Wells, 2021) and is endogenous to the gut microbiome. In the body, PPA is converted to propionyl-CoA, then ultimately is converted to succinyl-CoA, which is a substrate in the citric acid cycle (Berg et al., 2002).

PPA is also a common food preservative due to its antifungal properties (Jyonouchi et al., 2002). PPA can inhibit microorganisms via inducing an oxidative stress response, and fungal apoptosis (Yun & Lee, 2016). Yun And Lee (2016) characterized fungal apoptosis induced by PPA looking at PPA-treated *Candida albicans* cells, and observed accumulation of reactive oxygen species, activation of caspase (a cysteine protease that initiates apoptosis), and DNA fragmentation. They also discovered that the PPA-induced apoptosis was mediated by mitochondria. PPA altered the mitochondrial membrane potential, leading to intracellular calcium release and the release of cytochrome c from the mitochondria into the cytosol, causing the activation of caspases that ultimately lead to an apoptosis cascade (Yun & Lee, 2016). PPA is also found naturally in foods such as milk, yogurt, and cheese (Ho, Luo, & Adams, 2009), and is indicated as an effective food preservative, therefore, factors such as diet can have an impact on the amount of PPA in the body.

1.2.3 Beneficial roles of PPA

PPA plays several important physiological roles in the body. In the gastrointestinal tract, PPA aids in gastric and colonic smooth muscle contractions (McManus et al., 2002) and dilation of colonic arteries (Mortensen et al., 1990). PPA metabolism is associated in the production of glucose (Ringer,1912), and along with the SCFA butyrate, acts as the main energy substrate in epithelial cells (Heerdt, Houston & Augenkicht, 1997). Additionally, PPA is important for host immunity as it activates mast cells (Karaki et al., 2006), and research highlights this importance as deficiencies in PPA have been associated with increased risk

for allergies (Böttcher et al., 2000; Roduit et al., 2018) and asthma (Ivashkin et al., 2019). Due to PPAs weak acid nature, levels in the body can also affect levels in the brain, as it is able to cross the blood-brain barrier passively, or via monocarboxylate transporters (Van der hee & Wells, 2021). In the brain, PPA can affect central nervous system function, and influence neurotransmission, immune activation, lipid metabolism, and gene expression (Lobzhanidze et al., 2019).

1.2.4 Neurotoxic effects of PPA

Normal levels of PPA are necessary for proper homeostatic functioning, however, an excess of PPA can have neurotoxic effects including oxidative stress, and metabolic and immune disturbance, similar to what is reported in ASD and propionic acidemia (Lobzhanidze *et al*., 2020). Propionic acidemia is a heritable metabolic disorder in which PPA cannot be metabolized (Brusque *et al*., 1999). There is a mutation in the gene that codes for propionyl CoA carboxylase, causing the enzyme to malfunction or be defective, resulting in the build-up of PPA in the blood. According to Tian et al. (2020), PPA serum levels in healthy controls are 2.843 ± 0.10 mmol/L, and in propionic acidemia, serum levels can range from 1–5 mmol/L, and 1umol/g in the brain (Brusque et al., 1999). Some symptoms of propionic acidemia, such as developmental delays and cognitive deficits overlap with and clinically resemble ASD (Brusque *et al*., 1999). The propionic acid model of autism posits that neurotoxic levels of PPA elicit an ASD-like behavioural phenotype in rodents, especially social deficits, repetitive behaviour, and increased anxiety (Meeking *et al*., 2020; Shams *et al*., 2019; McFabe *et al*.,

2007, 2011, Benzaquen *et al*., 2010; Wah et al., 2019; Lobzhanidze *et* al., 2018; Lobzhanidze et al., 2020; Choi *et al*., 2018).

1.2.5 Evidence supporting PPAs involvement in ASD

Evidence for the involvement of PPA in ASD includes finding higher levels of *clostridia spp.*, the fermenting bacterial antecedent of PPA, in the feces of humans with ASD compared to healthy controls (Parracho *et al*., 2005). Researchers have also observed that mothers who take the mood stabilizer or anticonvulsant medication Valproic acid (VPA) during the first trimester of their pregnancy present an increased risk of their child developing ASD, as VPA has been shown to elevate PPA levels in both humans and animal models (Ornoy, 2009). Additionally, anecdotal evidence from parents of children with ASD suggests that with more PPA present in a child's diet (from refined wheat and dairy products), more severe behavioural symptoms are observed (Horvath et al., 1999).

1.2.6 Animal models

Central effects of PPA have been directly observed as brains from rodents treated with 4μl intracerebroventricular (ICV) infusions of 0.26M PPA have shown microglial and astroglial activation, fatty acid and mitochondrial dysfunction, elevated amounts of neurotoxic cytokines and markers of oxidative stress, and other changes consistent with findings in humans with ASD (; El-Ansary et al., 2018; MacFabe et al., 2007 ; Mepham et al., 2019;; Shams et al., 2019). Similar neuroinflammatory responses have been observed in vitro using rat

cell lines treated with PPA (Nankova et al., 2014). Along with central effects, behavioural effects are also observed in rodent models of PPA-induced ASD. Studies with direct central administration through ICV injections of 4µl of 0.26M PPA into the lateral ventricle have shown impairments in social behaviour including social approach and social interaction in both male adolescent and adult rats (MacFabe et al., 2007, 2011; Shultz et al., 2008).

Although PPA via the ICV route proves to be a good model, the systemic route of PPA via intraperitoneal (i.p) injections is more representative of an enteric source of PPA influencing behaviour (Shams et al., 2019). Several studies using repeated peripheral administration of 500mg/kg of PPA have also demonstrated behavioural symptoms that align with ASD such as abnormal social behaviour, increased anxiety, and hypoactivity (Benzaquen et al., 2010; Shams et al., 2009, 2018). These behavioural deficits were observed following chronic administration of PPA. However singular i.p doses as low as 175mg/kg have been found to elicit some social deficits and morphological changes in the amygdala of adolescent male rats (Lobzhanidze et al., 2020). There have also been reports of a dose-response curve where stronger effects of PPA were observed at higher doses for both central and peripheral injections in rats. Kamen et al. (2019) found that systemic doses of 500mg/kg reduced acoustic startle response magnitude more so than the dose of 250mg/kg . Meeking et al. (2020) found that rats showed more repetitive and stereotypic behaviours with ICV doses of 4µl of 0.26M PPA compared to 0.052 M PPA.

1.3 Sensory processing deficits in ASD relate to symptom severity

Another major aspect of ASD involves difficulties with sensory processing, so much so that the 5th edition of the Diagnostic Statistical Manual (DSM-5) now includes both hyper and hyposensitivity to sensory stimuli as key diagnostic factors of the disorder (American Psychiatric Association, 2013). People with ASD can have sensory processing issues across multiple modalities including vision, hearing, touch, olfaction, and gustation (Thye et al., 2018). Sensory processing and social deficits in ASD may reciprocally influence each other during a child's development (Gliga et al., 2014). A cross-sectional study examined associations between hyporesponsiveness and social communication outcomes in children with ASD and found that hyporesponsiveness to social and non-social stimuli predicts lower levels of language development and joint attention (where two people understand that they both share interest in an object or event) (Baranek et al., 2013). Hilton et al (2010) looked at sensory responsiveness as a predictor for the severity of social deficits in children with high functioning ASD and found that atypical sensory responsiveness and social deficits were highly correlated when neurotypical controls and high functioning ASD participants were aggregated. This suggests that in general, sensory processing deficits and social impairment are related.

1.3.1 Habituation deficits in ASD correlate with social deficits

An important feature of sensory processing is habituation, which occurs when there is a diminished response to a stimulus following repeated exposure (Jamal et al., 2020). This process is a form of learning, and has been observed

across species (Jamal et al., 2020). Ignoring irrelevant stimuli (i.e., habituation) is perceived to be a way in which filtering mechanisms in the brain prevent information overload (Fenckova et al., 2019). This allows for selective attention to occur, which increases focus towards relevant stimuli (Fenckova et al., 2019). Habituation learning is thought to be a prerequisite for higher cognitive functioning. This idea is supported by studies on infants wherein habituation during infancy correlates with cognitive performance in childhood (Kavšek, 2004). There is evidence to suggest that for individuals with neural deficits, such as ASD, habituation may be impaired (Kleinhans et al, 2009; Sinha et al., 2014; Jamal et al., 2020). A recent electroencephalograph human study by Jamal et al. (2020) compared habituation to repetitive stimuli of neurotypical (NT) children to those with ASD. They hypothesized that ASD may be associated with a reduction in sensory habituation and found that for both auditory (beep noise) and visual (checkerboard) stimuli, children with ASD displayed significantly less habituation compared to NT children (Jamal et al., 2020). A similar study with NT adults and adults with ASD looked at abnormal habituation to neutral facial cues in the fMRI (Kleinhans et al., 2009). They observed significantly reduced habituation in the ASD group compared to the NT group. They also found that in the ASD group, less habituation to the facial stimuli correlated with more severe social deficits in the participants (Kleinhans et al., 2009). These results suggest that reduced habituation may in part be a basis for social impairment in ASD (Kleinhans et al., 2009).

1.3.2 PPAs involvement in sensory processing deficits

PPA has been implicated in impairing sensory responsiveness as measured via the acoustic startle response (ASR) in rodent models (Foley et al., 2015; Kamen et al., 2018; Wah et al., 2019). The ASR is an involuntary reflexive response observed after a sudden pulse of a loud noise (Graham, 1975). Pre-pulse inhibition (PPI) is used to examine sensorimotor gating and occurs when a weak pre-pulse noise is presented immediately before the acoustic startle that is meant to weaken the ASR (Graham, 1975). Wah et al (2019) showed that peripherally injected PPA at 500mg/kg had a main effect on the ASR, where PPA treated rats demonstrated impairment in their startle response, as it was significantly lower than the control group, Kamen et al (2018) showed similar results with the same dosage. Wah et al (2019) also observed that PPA treated rats during adolescent and later as adults elicited a decrease in PPI, consistent with previous research (Foley et al., 2015). PPI is a measure that looks at the ability to filter out auditory stimuli through habituation (Wah et al., 2019), and since PPA decreased the rats' ability to modulate the startle response when exposed to a pre-pulse, PPA may be responsible for the inability to filter out irrelevant auditory stimuli. PPA treated rats have also shown a lack of habituation to their environment. In an open-field study, Brusque et al (1999) observed that rats treated with chronic subcutaneous injections of PPA failed to habituate to the open-field, as locomotor variables did not decrease upon re-exposure, suggesting that peripheral PPA affects sensory processing. It was of interest in this study to determine if PPA could also alter sensory processing for olfactory stimuli.

1.4 Olfactory behaviour in rodents

In the animal kingdom, especially for rodents, communication occurs largely via olfactory signals (Weller, 1998). A crucial determinant of social behaviour in rodents is odorant communication, where detecting olfactory social cues allows the animal to gather information from conspecifics, including potential mates, and decide whether to approach or avoid them (Arakawa, Cruz, & Deak, 2011). For rodents, olfactory behaviour can be measured by sniffing behaviour. Sniffing is a rhythmic orofacial motor activity characterized by the voluntary inhalation of air for the purpose of sampling an odour (Welker 1964). Rats have resting sniffing frequencies around 2Hz, and significantly increase the frequency to 4-12Hz when investigating novel odour stimuli (Uchida & Mainen, 2003; Kepecs, Uchida & Mainen, 2007). There is also evidence of odour habituation, as sniffing frequencies decrease once the rodent becomes familiar with the odour (Arubuckle et al., 2015; Sundberg et al., 1982).

1.5. PPAs potential role in odour habituation, and social deficits in rodents

The studies discussed above show that PPA from the gut correlate with the development of social deficit. It is also evident that children and adults with ASD have difficulties habituating to social and sensory stimuli, which may be a factor in social impairment. However, to date, there has been no study assessing the role of PPA in habituation to sensory social cues. Sensory processing appears to be impaired in rodent models of ASD as PPA treated rats show impaired auditory filtering (Foley et al., 2015; Kamen et al., 2018; Wah et al., 2019), and a lack of habituation to the open-field (Brusque et al., 1999). When social odours and non-

social odours are presented to rats, PPA may also affect olfactory sensory processing and result in lack of habituation and/or social recognition. Abnormal social behaviour has been observed in PPA treated rats (Shultz et al., 2008; Foley et al., 2014; Shultz et al., 2015; Shams et al., 2019), and this may be a result of impaired olfactory habituation.

1.5.1 Hypothesis and goals of the present study

I hypothesized that social deficits seen in PPA treated rats are due to a lack of habituation to social odours. There were two main goals of this study:

1. Measure habituation to social and non-social odour cues in rats treated with PPA and controls.
2. Measure dishabituation to social and non-social odour cues in rats treated with PPA and controls.

Other goals of this study were to:

3. Measure affective behaviour variables for PPA treated rats versus controls.
4. Measure locomotor activity variables for PPA treated rats versus controls.
5. Quantify behaviour and locomotor activity using automated and manual scoring and compare results of both methods.
6. Determine the effects of PPA and the vehicle control on peripheral body temperature and body weight.

I wanted to measure habituation to an odour followed by dishabituation to a novel odour, and to examine the effects of PPA on odour habituation, dishabituation, and affective behaviour. Affective variables include behaviours that are reflective of a negative or positive mood state, including contentment, anxiety, and fear (Kim, Kim & Shin, 2021). I predicted that rats treated with 500mg/kg of PPA would show increased anxiety-like behaviour, impaired social recognition, and therefore a lack of habituation and or dishabituation to social odours, but not for non-social odours. These predictions are based on previous research that shows decreased social interest and social activity with rats treated with neurotoxic ICV doses of 4µl of 0.26 M PPA, and systemic doses of 500mg/kg (MacFabe et al., 2007, 2011; Shultz et al., 2008; Choi et al., 2018). To study social recognition and habituation, typically a habituation-dishabituation paradigm is used whereby an animal is exposed to stimuli from a conspecific for one or multiple trials (Gheusi et al., 1994). Removal and re-introduction of the stimulus typically results in reduced social investigation by the experimental animal (habituation). Conversely, if a novel stimulus is introduced and social memory persists, this novelty will result in a higher level of investigation (dishabituation). By measuring locomotor activity, the rats' movement and exploratory behaviour could be tracked. In previous studies, PPA induced hypoactivity, therefore locomotor measures were also important in determining the effectiveness of the drug (Ossenkopp et al., 2012; Shams et al., 2019; Wah et al., 2019; Lobzhanidze et al., 2020).

The current study examined the effects of PPA on habituation to olfactory stimuli, specifically social and non-social odours using adult male rats. Males were used in behavioural experiments based on the male predominance in ASD (Foley et al., 2014), and that male rats are more likely to engage in social interaction than female rats (Thor & Holloway, 1984; Foley et al., 2014).

Additionally, I wanted to develop an automated approach to quantify investigative behaviour towards odour and to see if automated variables were as sufficient at detecting these behaviours as manual scoring. Repetitive behaviours were also scored both manually and automatically to observe efficacy of automated results. Creating an accurate automated paradigm for tracking odour investigative behaviour and repetitive behaviours would provide a more efficient and faster process when analyzing the results of future experiments. The final goal of this study was to look at the effects of PPA on body temperature and body weight. Since PPA is a bacterial product (van der Hee & Wells, 2021), and is able to induce an inflammatory response (Karaki et al., 2006), it may elicit malaise by indirectly increasing body temperature and decreasing body weight, similar to the body's immune response to pathogenic bacteria. To provide insight on the animals' overall health and to see if PPA exerts its effects by inducing a sickness response, body temperature and body weight were monitored before and after injections.

2.0 Material and Methods

2.1 Subjects

Sixty-four adult male Long Evans rats were used (226-250g, ~60 days of age; Charles River, Kingston). Fifty-two rats receiving the same experimental treatment were pair-housed. Twelve rats were single-housed and used as odour donors and did not receive any treatment. All animals were housed in standard polypropylene cages (26 cm × 48 cm × 21 cm) in a temperature-controlled colony room (20 ± 1◦C) on a 12:12h light: dark cycle with food (ProLab RHM3000 rat chow) and tap water available ad libitum, except during test sessions. All procedures were carried out during the light phase of the light:dark cycle (10:00–16:00 hr). All procedures and experimentation were approved and carried out according to the guidelines set by the Canadian Council on Animal Care and approved by the institutional animal care committee.

2.2 Drugs

Sodium propionate (PPA, P1880, Sigma-Aldrich, St. Louis, MO, USA) at a dose of 500 mg/kg was dissolved in 0.1 M phosphate buffered saline (PBS) and buffered to pH of 7.5 using HCl. The dose of PPA was based on the results of prior studies showing that 500mg/kg had significant effects on social behaviour in adult male Long Evans rats (Shams et al. 2018; Wah et al., 2019). Rats were randomly assigned to receive either sodium propionate/propionic acid (500mg/kg) or a vehicle control solution of PBS via i.p. injections.

2.3 Odours

The rats were randomly assigned to be exposed to either non-social or social odours. The social odours cues consisted of 7g of soiled bedding from the home cages of single-housed unfamiliar rats (odour donors) collected 2-3 days after their bedding was changed. Social odours were presented in a (8.5 cm x 1.5cm) petri dish covered with a wire mesh with 1cm squares to prevent digging. The non-social odour cues consisted of vanilla and almond extract. Both non-social odours were diluted with deionized water in a 1:10 manner respectively, with 1 ml of the solution dripped onto 7g of clean bedding in a petri dish. This presentation was based on previous studies that have used similar paradigms (Seillier & Giuffrida, 2015).

2.4 Experimental procedure

After arrival, rats were left undisturbed for one week to acclimate to the facility, then handled for three consecutive days and numbered on their tails with a non-toxic permanent marker for identification. Prior to testing, rats were habituated to the experimental apparatus for thirty minutes for two consecutive days. Next, rats were weighed and pre-loaded with 500mg/kg of PPA or PBS (i.p.) for four consecutive days before test days, similar to the procedures of Shams et al. (2019). For this experiment, pre-loading with PPA was necessary to mimic a pre-existing excess of PPA, similar to what has been found in individuals diagnosed with ASD (Wang et al., 2012). During the pre-loading period, peripheral body temperature was captured using a non-invasive infrared (IR) camera (FLIR E75, FLIR Systems, Wilsonville, OR, USA) hovering over the base of the rats' tails

before and 30 minutes after each injection on each day (Katz et al., 2012). On the baseline day, rats were injected (i.p.) with PPA or PBS and all groups were exposed to clean bedding for 10 minutes in the OF to monitor investigative behaviour towards a familiar neutral odour stimulus. Next, the rats were injected (i.p.) with PPA or PBS and exposed to the first odour cue (vanilla extract or unfamiliar [stranger] rat odour) for 10 minutes for three consecutive test days to observe habituation. On the fourth test day, rats were injected (i.p.) with PPA or PBS and then exposed to a novel odour (almond extract or unfamiliar rat) to test their ability to dishabituate from the original odour and recognize the unfamiliarity of the new sensory cue. The experimental timeline is described in Figure 1. Groups were as follows: PBS-non-social (n = 14, 7 pairs), PBS-social (n = 12, 6 pairs), PPA-non-social (n = 12, 6 pairs) and PPA social (n = 13, 7 pairs, one rat omitted due to technical complications). The odour quadrant was counterbalanced, as the petri dish alternated corners each day to avoid place preference.

2.5 Apparatus

2.5.1 Automated analysis

Eight automated VersaMax Animal Activity Monitors (42×42×30 cm; Accuscan Model RXYZCM-16, Columbus, OH) served as Ofs to record the locomotor activity of individual animals (Figure 2 a). The Versamax system can detect the horizontal and vertical activity in a Plexiglas chamber, in addition to the duration of time the rat spends close to or away from a target via 16 infrared beams surrounding the perimeter of the OF at 7cm and 18cm above the floor. A

clear plexiglass partition with an opening at the base (10cmx15cmx10cm) was used to separate the OF into 2 chambers, while allowing the animal to traverse between chambers, making it suitable for a 2-chamber test. The Versamax software further separates the OF into 4 quadrants (Figure 2 b). For this experiment, there was one odour quadrant and three "no odour" empty quadrants (see Figure 2 b). The petri dish was fixed with Velcro onto one corner (corners alternated daily) of the OF so the rat could explore the odour stimulus without perturbing the petri dish, this area was designated as the odour quadrant. During habituation testing, a rat was placed in the OF in the opposite quadrant of the petri dish and was free to investigate for 10 minutes. The automated variables detected by the Versamax were as follows:

- *Entries into the odour quadrant*: The number of times a rat entered the quadrant with the odour stimulus.
- *Total vertical activity*: The cumulative number of times a rat interrupted the vertical infrared sensors (i.e., by rearing/being on its hind legs).
- *Total horizontal activity*: The cumulative number of times a rat interrupted the horizontal infrared sensors.
- *Total distance travelled*: The cumulative horizontal distance travelled in cm.
- *Stereotypy*: When the animal interrupts the same infrared beam or set of beams repeatedly with no goal that may reflect negative welfare or affective state; an affective variable (Novak et al., 2016).

- *Stereotypy count (Total STRCNT):* The cumulative number of stereotypical movements, or single beam breaks the infrared sensors detected while the animal exhibited stereotypy. This movement typically occurs during grooming and/or head bobbing.
- *Stereotypic episodes (Total STREP):* The number of times the monitor detects stereotypic episodes. Stereotypic episodes are separated by a break of at least one second.

*Note: Automated variables provided data for each zone ("odour quadrant" and "no odour zone"). All automated measures used centroid detection, where the centre of the animal's body had to be in a quadrant to be detected.

2.6 Video analysis & manual scoring

The Plexiglas chamber within the Versamax had a transparent Plexiglas lid with air-holes, allowing for the sessions to be videotaped for later manual scoring. A video camera (Canon HD Camcorder VIXIA HF W10, JVC Everio Camcorder GZ- MG360, Sony Handycam DCR-DVD201) was mounted onto a tripod placed directly in front of the Versamax chamber and angled downward towards the OF to provide a full view of both chambers. The frequency of social (approach and investigative sniffing) and non-social (self-grooming) behaviours were manually scored from video footage by the thesis writer blindly. This aggregation into social and non-social categories was based on previous studies (Ossenkopp & Mazmanian, 1985). Definitions for these behaviours are as follows:

- *Frequency of approach*: The cumulative number of times the animal approached the petri dish within 5cm of its nose.
- *Frequency of sniffing*: The cumulative number of times the animal sniffed the odour within 5 cm of the petri dish. For this variable, the rat had to be oriented towards the petri dish with the tip of its nose. Each sniff within a wire mesh square was scored as one.
- *Self-grooming*: The cumulative number of times the animal switched between licking/cleaning its face/head to its abdominal or groin area, to its paw, to its tail). Grooms were also counted if there was a break of over 1 second between licking activity.

Accuracy of automated variables for the automated system was previously examined by Sanberg (1985), who showed that when the animal entered a quadrant in the open-field on video, the automated system detected movement in the same quadrant.

2.7 Statistical Analysis

Statistical analyses were performed using IBM SPSS Statistics 24. Data from test days 1-4 were analyzed for main and interactions effects using a repeated measures 3-factor analysis of variance (ANOVA) with Drug (2 levels: PPA or PBS) and Odour (2 levels: social or non-social) as the between subject factors, and Test Day as the within subjects factor (4 levels: test day 1, test day 2, test day 3, test day 4, or 5 levels: baseline day, test day 1, test day 2, test day 3, test day 4),). Baseline data were analyzed using a univariate ANOVA, with a between-subject factor of Drug (2 levels: PPA or PBS). All tests were carried out

using a significance criterion of $\alpha=0.05$. Greenhouse-Geisser corrections were applied where Mauchly's test of sphericity was violated. LSD post-hoc pairwise comparisons were used to look at significance levels if main effects or interactions were detected. Pearson correlations were calculated to assess the reliability of the automated versus manually scored variables measured across test days. Correlation matrices were created to observe the Pearson correlation of the automated variable, entries into the odour quadrant, with manually scored variables including Frequency of Approach and Frequency of Sniffing for each habituation day (test days 1-3) to find similarities in odour investigation. The Pearson correlation of the manually scored variable, self-grooming, was put into correlation matrices along with stereotypy measures for each habituation day (test days 1-3) to look for similarities in analysis of stereotypic behaviour. To further examine PPAs effects on sensory processing to social odours, a multivariate analysis was performed specifically focusing on social groups, to look at the relationship between PPA and multiple habituation variables. Test day 3 results were compared to test day 1 results to observe habituation. Another multivariate analysis was performed, also focusing on only social groups, to look the relationship between PPA and multiple dishabituation variables, by comparing test day 4 results to test day 1 results.

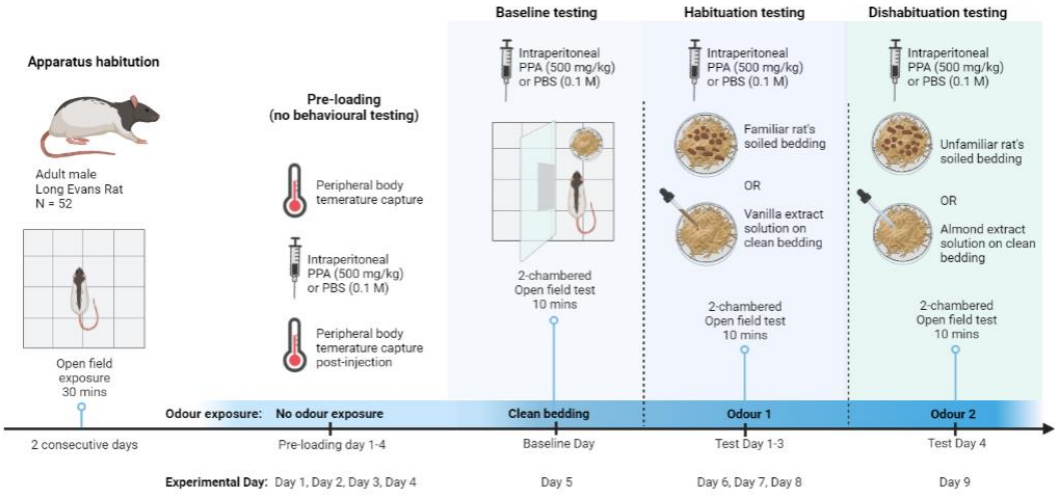


Figure 1. Experimental Timeline. Rats first habituated to the open-field apparatus for 30 minutes for 2 consecutive days. On the pre-loading days, rats body temperature was recorded before and 30 minutes after intraperitoneal injections of PPA (500mg/kg) or the vehicle control, PBS. Next, rats were given daily injections (i.p.) and placed into the open-field for 10 minutes along with the odour stimulus. On the baseline day, all rats were exposed to clean bedding. For the test days, rats were separated into 4 groups, PBS + non-social odour, PPA + non-social odour, PBS + social odour and PPA + social odour. Non-social odours were 1 ml of diluted vanilla and almond extract dripped onto 7g of clean bedding. Social odours were 7g of soiled bedding from unfamiliar (stranger) rats. During habituation, the rats were exposed to the same odour for 3 consecutive days. On the 4th test day during dishabituation, a novel odour was introduced. Figure created with BioRender.com.

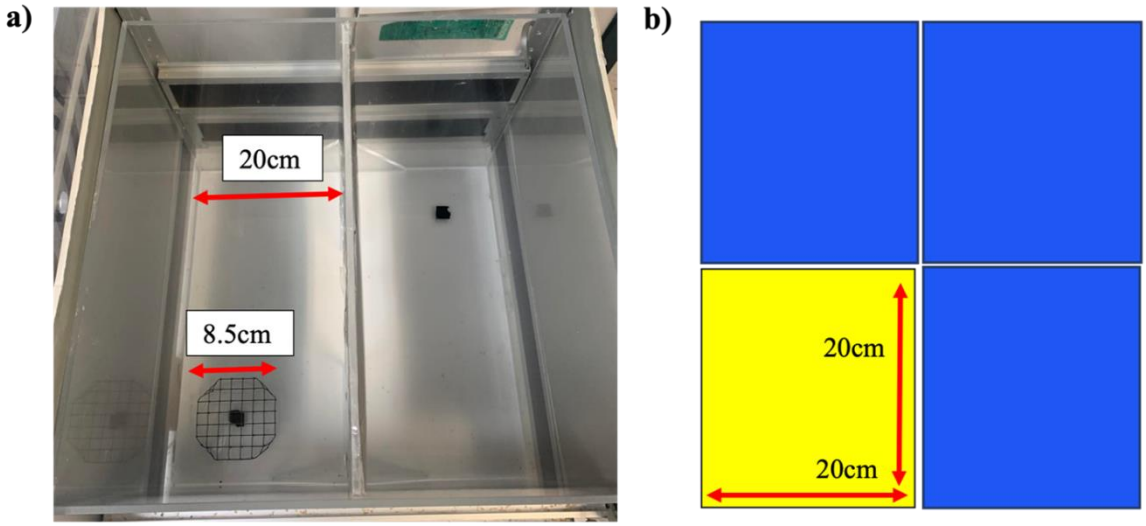


Figure 2: Apparatus. A) The VersaMax open-field chamber. A partition separated the open field into 2 chambers, each 20 cm wide. The petri dish was placed in the corner of one chamber and was 8.5x8.5cm wide. The odour quadrant measured 20cm by 20cm. B) The odour quadrant (yellow) measuring 20x20cm versus the no-odour zones (blue) which was made up of 3 identically sized quadrants

3.0 Results

3.1 Locomotor activity variables

3.1.1 Total vertical activity

On the baseline day, there was an effect of Drug on total vertical activity, $F(1,48) = 9.081$, $p = .004$, where PPA treated rats reared significantly less than the rats given PBS (Figure 3 a). No significant main effect of Odour ($F(1,48) = 0.185$, $p = .669$), or significant interaction effect of Drug and Odour ($F(1,48) = 0.007$, $p = .935$), were observed. A main effect of PPA was found across test days for total vertical activity, $F(1,47) = 5.635$, $p = .022$. Post hoc analysis showed significant drug group differences across the test days, as seen in Figure 4 a. No significant main effect of Odour ($F(1,48) = 1.308$, $p = .258$), or significant interaction effect of Drug and Odour ($F(1,48) = 2.090$, $p = .155$), were observed.

3.1.2 Total distance travelled

On the baseline day there was a significant Drug effect, $F(1,48) = 11.928$, $p = .002$, where PPA treated rats travelled significantly less distances in the OF than PBS treated rats (Figure 3 b). No significant main effect of Odour ($F(1,48) = 1.308$, $p = .258$), or significant interaction effect of Drug and Odour ($F(1,48) = 2.090$, $p = .155$) were observed. Across test days, there was also main effect of PPA, $F(1,47) = 8.212$, $p = .006$. Post-hoc analysis revealed significant drug group differences on all the test days (Figure 4 b). There was also a significant main effect of test day, $F(1,3) = 3.981$, $p = .011$, and post-hoc analyses revealed test day differences on test day 1 and 3, which is an indication of habituation as

distance travelled significantly decreased on test day 3 (p = .004). Test day 1 was also different from test day 4, where distance travelled decreased on test day 4 (p = .004). There were no significant effects of Odour (F(1,48) = 1.308, p = .258), or any significant interactions between Drug and Odour (F(1,48) = 2.090, p = .155).

3.1.3 Total horizontal activity

On the baseline day there was a significant main effect of Drug, F (1,48) = 6.560, p = .014. PPA treated rats displayed less horizontal beam breaks than PBS treated rats. The ANOVA for total horizontal activity across test days also showed a significant main effect of Drug, F (1,47) = 5.193, p = .027. Post hoc analysis revealed significant group differences on all test days (Figure 4 c), where PBS rats experienced more horizontal activity than PPA rats. There were no significant effects of Odour F(1,47) = 1.243, p = .270, or any significant interactions between Drug and Odour F(1,47) = 3.097, p = .085.

3.1.4 Locomotor activity results summary

PPA treated rats displayed significant hypoactivity compared to the vehicle control rats for all vertical and horizontal measures in the open-field. The lack of odour and interaction effects suggests that odours had no effect on these locomotor variables, and odours did not influence drug effects. There was an overall significant decrease in total distance travelled from test day 1 to 3, but no significant differences in habituation across groups. There were also no significant effects of PPA on dishabituation to the novel environment, as test day 3 results were not significantly different than test day 4.

3.2 Odour investigation

3.2.1 Entries into the odour quadrant

For baseline Entries into the Odour Quadrant, there was a significant main effect of Drug, $F(1,48) = 4.360$, $p = .042$. PPA groups showed significant decreases in the number of entries into the odour quadrant compared to the PBS groups. However, Odour had no significant effect on odour quadrant entry, $F(1,48) = .019$, $p = .890$, and no significant interactions were found between Drug and Odour, $F(1,48) = 1.211$, $p = .277$. PPA treated rats also showed a significant decrease in entries into the odour quadrant on test days, $F(1,47) = 4.197$, $p = 0.465$. Post hoc analyses revealed significant drug treatment group differences across test days, where PPA rats consistently entered the odour quadrant significantly less than the control groups (Figure 5). Again, the ANOVA revealed no significant main effect for Odour, $F(1,47) = .061$, $p = .806$, and no significant interaction between Drug and Odour, $F(1,47) = 71.356$, $p = .087$.

3.2.2 Approach towards the odour stimulus

On the baseline day, a significant main effect of Drug was found, such that PPA treated rats approached the odour stimulus significantly less than PBS treated rats, $F(1,48) = 7.799$, $p = .007$. Consistent with previous measures, there were no significant main effects of Odour, $F(1,48) = 3.208$, $p = .080$, or interaction effects between Drug and Odour, $F(1,48) = 1.411$, $p = .241$. The repeated measures ANOVA for approach across test days suggested a significant main effect of Drug on the test days, $F(1,47) = 11.236$, $p = .002$. Post hoc analysis revealed that PPA treated rats approached the odour stimulus less often on all test days (Figure 6).

There was also a significant main effect of test day, $F(1,47) = 5.027$, $p = .002$, and post-hoc analyses revealed significant differences on test day 1 and 3, where approaches towards the odour stimulus significantly decreased on test day 3 ($p = .001$).

3.2.3 Frequency of sniffing

A significant main effect of Odour was found across test days $F(1,47) = 17.992$, $p < .001$. Post hoc analyses showed odour group differences where rats exposed to social odours sniffed the odour stimulus significantly more across test days (Figure 7 a). There was also a main effect of test day, $F(1,3) = 7.547$, $p = <.001$. Post hoc analyses showed sniffing differences on test days 1 and 2 ($p = .004$), test days 1 and 3 ($p < .001$) and test days 1 and 4 ($p = .002$). On all subsequent days from test day 1, overall rat sniffing decreased. No significant effect of Drug, $F(1,47) = .829$, $p = .367$, or interactions between Drug and Odour, $F(1,47) = .265$, $p = .609$, were found.

3.2.4 Results summary for odour investigation

PPA treated rats approached the odour stimulus less and entered the odour quadrant less often than the control vehicle treated animals. The absence of odour and interaction effects implies that odours did not influence drug effects for measures of approach and entries towards the odour. Frequency of sniffing was only affected by type of odour, as social odours were sniffed significantly more for both PPA and PBS rats, however, drug treatment had no significant effects. Additionally, there were no significant effects of PPA on habituation or dishabituation for all measures of odour investigation. No groups (including

controls) showed significant dishabituation to the odours as there were no significant increases in odour investigation on the fourth test day.

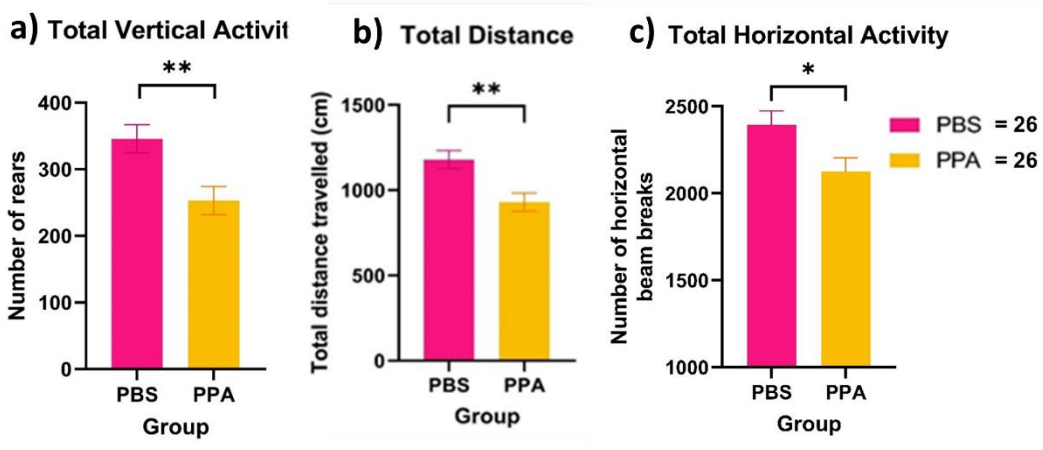


Figure 3. Baseline locomotor activity in the open-field. A) Total vertical activity. PBS rats reared significantly more than PPA rats (**p = .004). B) Total distance. PBS rats travelled significantly larger distances across the open field chamber than PPA rats (**p = .002). C) Total horizontal activity. PBS rats caused significantly more horizontal beam breaks than PPA rats (*p = .014). Data represented as mean ± SEM

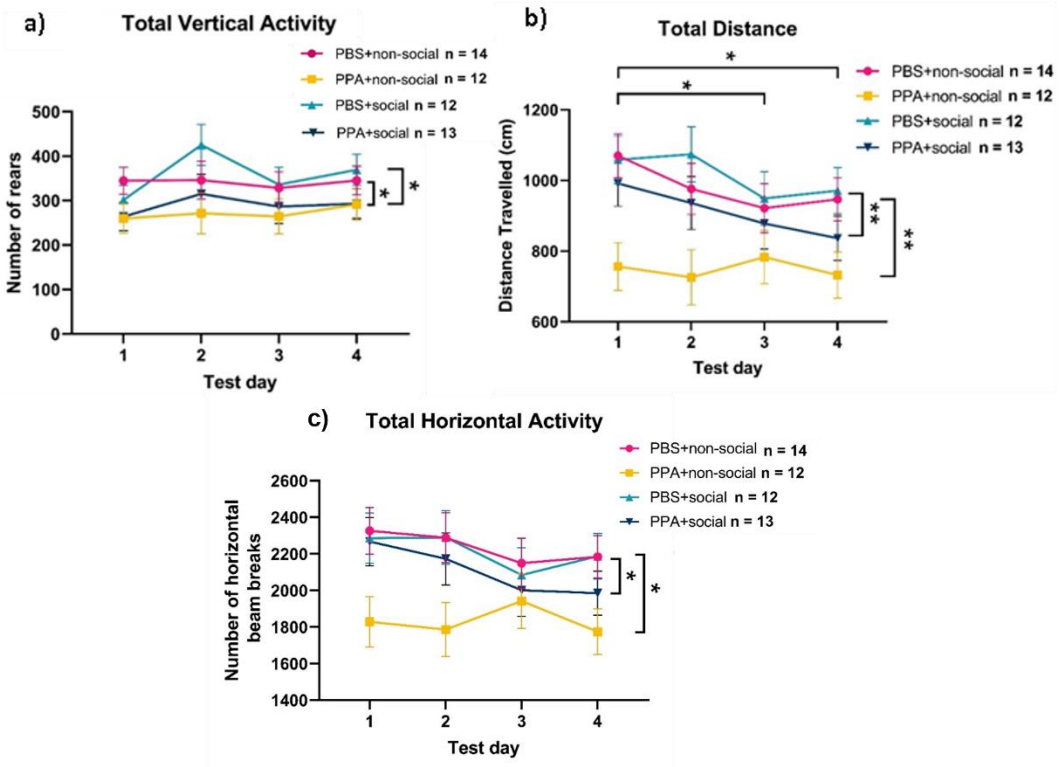


Figure 4. Locomotor activity in the open-field across test days. A) Total vertical activity. PBS rats reared significantly more than PPA rats across test days (**p* = .022). B) Total distance travelled. PBS rats travelled significantly larger distances than PPA rats across test days (***p* = .006). Test day 1 and 3 were significantly different from one another (***p* = .004), as well as on test day 1 and 4 (***p* = .004). Odour had no significant effect on motor activity. Data represented as mean ± SEM. C) Total horizontal activity. PPA groups displayed significantly less horizontal beam breaks than PBS groups (**p* = .027). Odour had no significant effects. On test day 4, PBS groups displayed an increase in horizontal activity when the novel odour was presented, and PPA groups displayed a decrease in horizontal activity

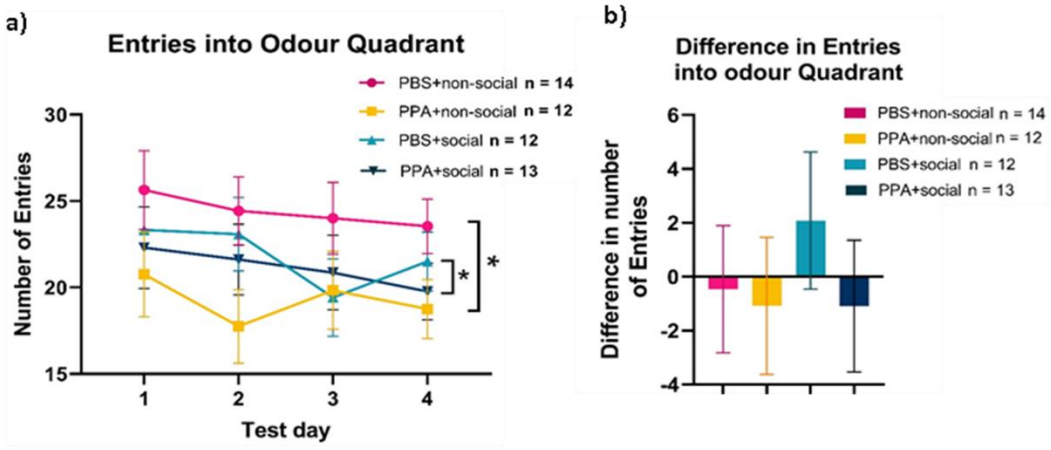


Figure 5. Entries into the odour quadrant. A) Entries into the odour quadrant across test days. PPA groups entered the odour quadrant significantly less often than the PBS groups (*p = .046). Odour had no significant effects. B) Difference in entries into the odour quadrant on test day 4 versus test day 3 (calculated by test 3 data subtracted from test day 4 data). Most groups experienced a decrease in entries into the odour quadrant when the novel odour was presented, except for the PBS+social group which showed an increased in the number of entries. Data represented as mean ± SEM.

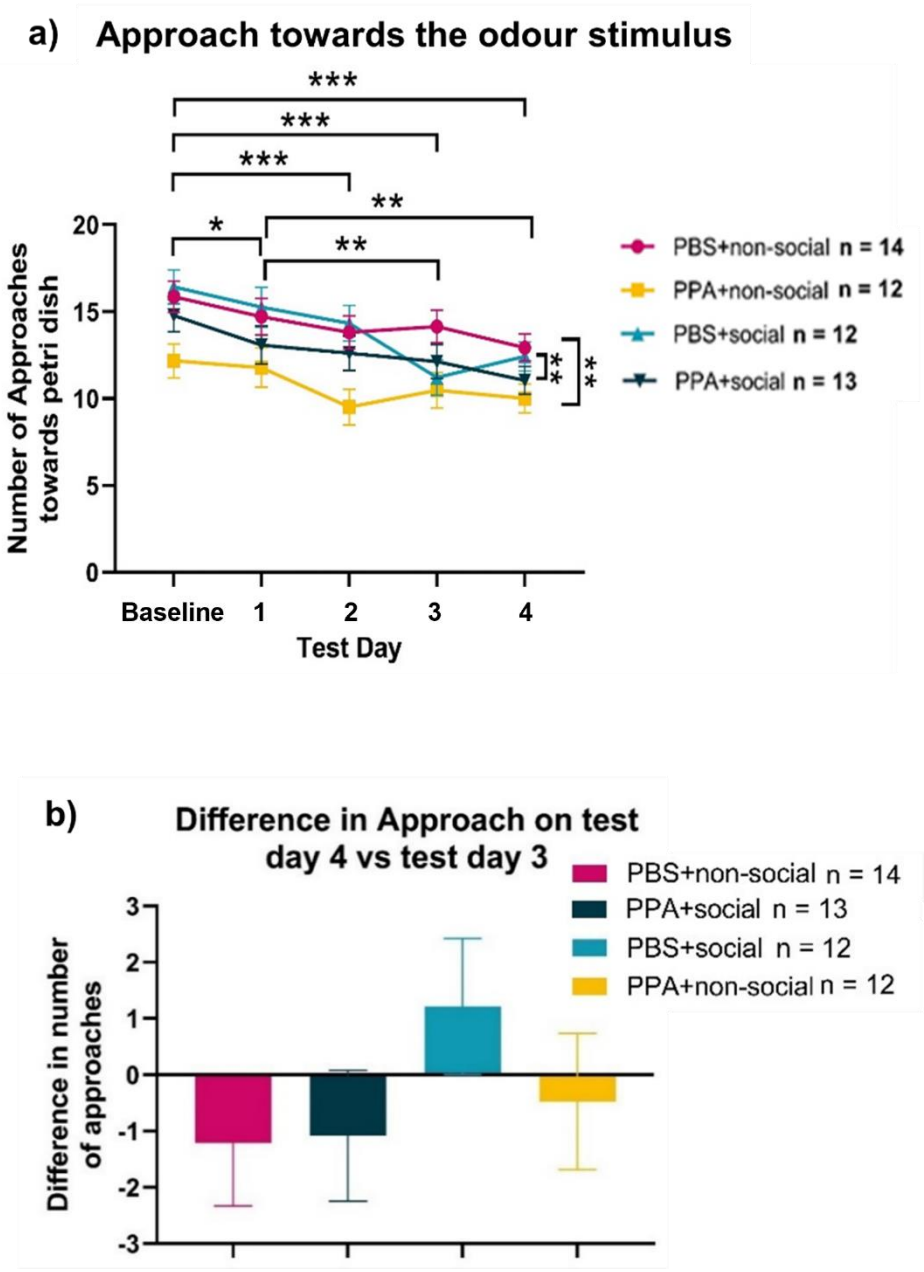
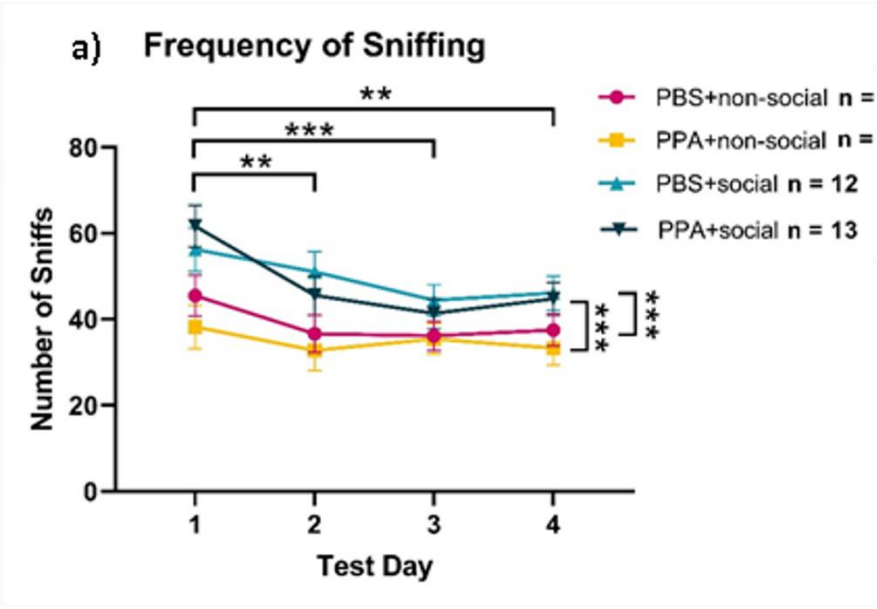


Figure 6. Frequency of approach. A) Approach towards the odour stimulus (petri dish) across test days. PBS groups approached the odour stimulus significantly more than the PPA groups. Odour had no significant effects. Baseline day was significantly different than test day 1 (*p = .037), test day 2 (**p = .001), test day 3 (***p < .001), and test day 4 (***p < .001). Test day 1 was significantly different than test day 3 (**p = .001) and test day 4 (**p = .002). B) Difference in approach frequency towards the odour stimulus (petri dish) on test day 4 versus test day 3 (calculated by test 3 data subtracted from test day 4 data). All groups except for the PBS+social group experienced a decrease in approach towards the odour stimulus when the novel odour was presented. The PBS+social group increased the number of approaches on the fourth test day. Data represented as mean ± SEM.



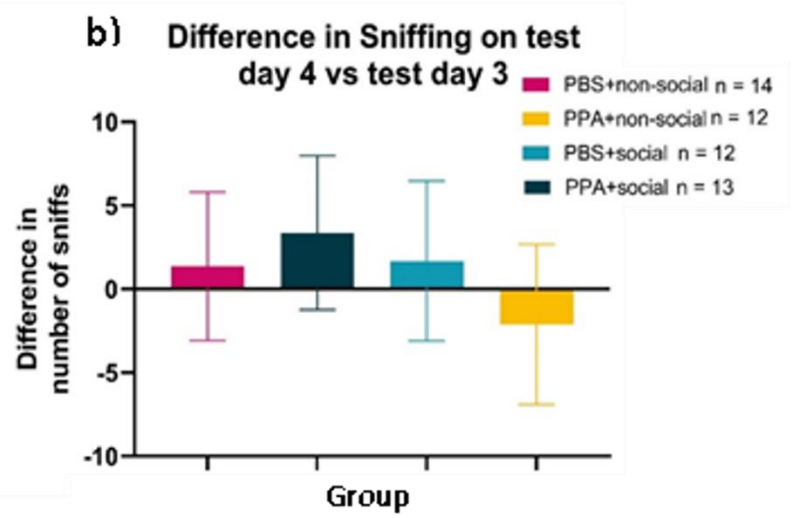


Figure 7. Frequency of sniffing. A) Frequency of sniffing the odour stimulus (petri dish) across test days. Odour had a significant effect as social odour groups sniffed the petri dish significantly more across test days. Sniffing from test day 1 was significantly different from test days 2 (**p = .004), 3 (***p < .001) and 4 (**p = .002). B) Difference in sniffing frequency of the odour stimulus (petri dish) on test day 4 versus test day 3 (calculated by test 3 data subtracted from test day 4 data). All groups experienced an increase sniffing frequency when the novel odour was presented, except for the PPA+non-social group that showed a decrease in sniffing frequency on the fourth test day. Data represented as mean ± SEM.

3.3 Affective Variables

3.3.1 Self-grooming

Video analysis of self-grooming showed a significant Drug effect, $F(1,47) = 6.194$, $p = .016$, where PPA treated rats self-groomed significantly more than PBS treated rats. Post-hoc analyses revealed significant increases in self-grooming for PPA groups across both baseline and test days (Figure 8 a).

3.3.2 Stereotypy

For the automated measures of stereotypy across baseline and test days, the ANOVA revealed no significant differences between drug treatments for stereotypic episodes, $F(1,46) = 3.741$, $p = .059$, and no significant effect of Odour ($F(1,46) = 1.110$, $p = .298$) or interaction effect of Drug and Odour ($F(1, 46) = 3.571$, $p = .065$). Similarly, for the number of stereotypical movements across baseline and test days, the ANOVA revealed no significant effect of Drug, $F(1, 46) = 2.073$, $p = .157$, Odours, $F(1,46) = 2.876$, $p = .097$, and no interaction effects between Drug and Odour, $F(1,46) = 1.125$, $p = .294$ (see Figure 9).

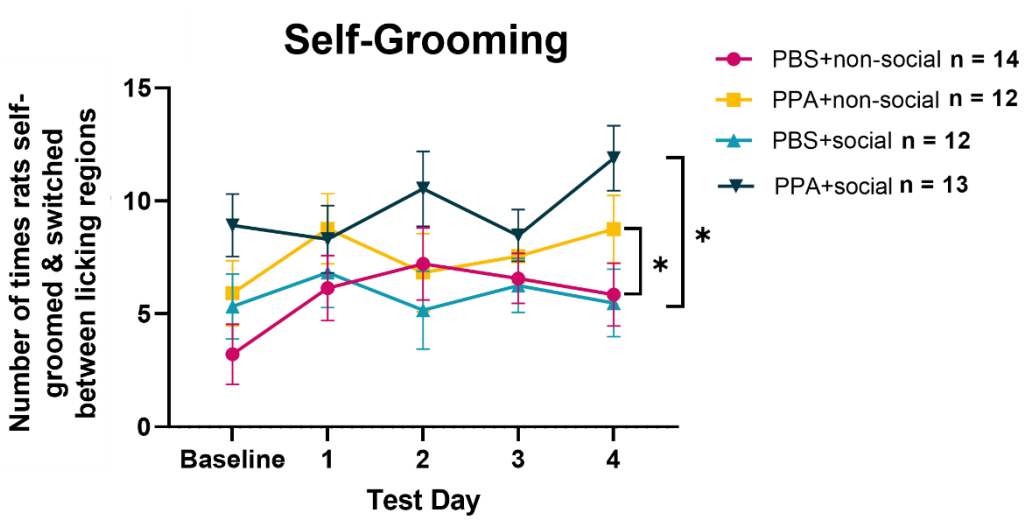


Figure 8. Self-grooming across baseline and test days. PPA treated rats self-groomed significantly more than PBS treated rats across test days (*p = .016). PPA groups increased self-grooming on the fourth test day, and PBS groups decreased self-grooming. Odour had no significant effect on self-grooming. Data represented as mean ± SEM.

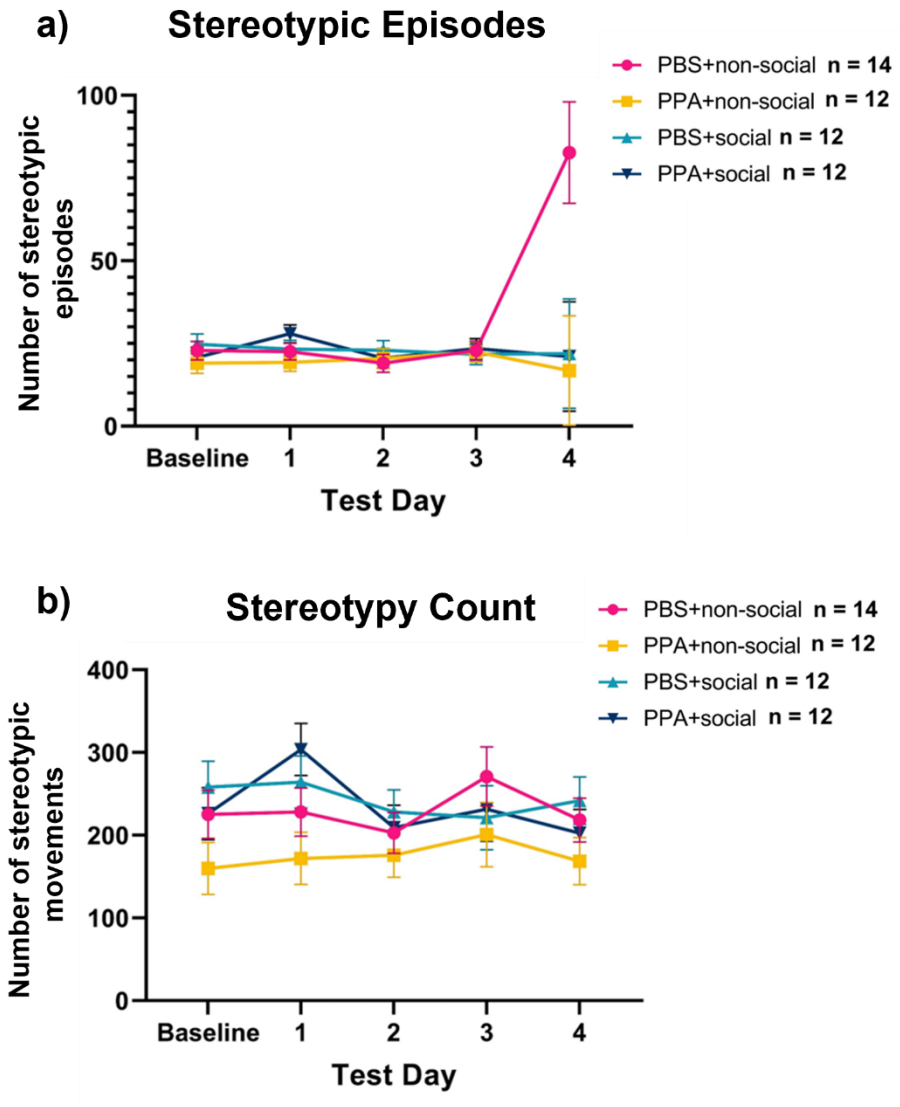


Figure 9. Stereotypic behaviour. A) Number of Stereotypical Episodes. Drug and Odour had no significant effects on number of stereotypical episodes. B) Stereotypy Count. Drug and Odour had no significant effects on stereotypy count (number of stereotypical movements). Data represented as mean ± SEM.

3.4 Multivariate analyses

Further analyses were conducted to assess if PPA social groups habituated and dishabituated differently than PBS social groups. To examine PPAs relationship to various habituation measures using only social groups, a multivariate analysis was conducted with drug as the independent variable, and measures of habituation for the independent variables, including frequency of approach, entries into the odour quadrant, frequency of sniffing, and total distance travelled. Results showed that frequency of approach for PPA and the vehicle were not significantly different from one another (p = .063). For entries into the odour quadrant, PPA and the vehicle were not significantly different from one another, (p = .463). Similarly, for frequency of sniffing and total distance travelled, social groups of PPA and PBS rats were not significantly different from each other, as p = .256 and p = .956, respectively (see Table 1).

A second multivariate analysis for social groups was conducted using drug as the independent variable, and measures of dishabituation as the dependent variables. There were no significant differences between social groups of PPA and PBS rats for all dishabituation measures as p = .168 for frequency of approach, p = .452 for entries into the odour quadrant, and p =.276 for frequency of sniffing (see Table 2).

Table 1. Multivariate analysis of habituation measures between PPA and the vehicle for social groups only (calculated by test day 3 - test day 1).

Dependent Variable	(I) Drug	(J) Drug	Mean Difference (I-J)	Std. Error	Sig.[a]
HabApproach	PBS	PPA	-3.087	1.583	.063
	PPA	PBS	3.087	1.583	.063
HabEntries	PBS	PPA	-2.468	3.306	.463
	PPA	PBS	2.468	3.306	.463
HabSniff	PBS	PPA	8.386	7.194	.256
	PPA	PBS	-8.386	7.194	.256
HabDistance	PBS	PPA	4.276	77.447	.956
	PPA	PBS	-4.276	77.447	.956

Table 2. Multivariate analysis of dishabituation measures between PPA and the vehicle for social groups only (calculated by test day 4 - test day 3).

Dependent Variable	(I) Drug	(J) Drug	Mean Difference (I-J)	Std. Error	Sig.[a]
DiffApproach	PBS	PPA	2.296	1.612	.168
	PPA	PBS	-2.296	1.612	.168
DiffDistance	PBS	PPA	63.254	82.619	.452
	PPA	PBS	-63.254	82.619	.452
DiffEntries	PBS	PPA	3.173	2.843	.276
	PPA	PBS	-3.173	2.843	.276

3.5 Correlation matrices

3.5.1 Odour investigative measures

Entries into the odour quadrant, frequency of sniffing and frequency of approach strongly correlated with one another across habituation days. On test day 1, entries and approach showed a strong correlation, $r = .712$, $p < .001$, as well as entries and sniffing, $r = .381$, $p = .006$, and approach and sniffing, $r = .454$, $p = .001$ (see Table 3). On test day 2, entries and approach strongly correlated, $r = .818$, $p < .001$, as well as entries and sniffing, $r = .340$, $p < .015$, and approach and sniffing, $r = .534$, $p < .001$ (Table 4). Lastly on test day 3, entries and approach correlated at $r = p < .001$, entries and sniffing correlated at $p = .017$, and approach and sniffing correlated at $p = .028$ (Table 5). These correlations suggest that the automated system adequately reflects what manual scoring of odour investigation shows.

3.5.2 Repetitive behaviour measures

The persistence of repetition of specific movements, or repetitive behaviours, was examined using stereotypy and self-grooming variables. Both automated stereotypy measures and the manually scored self-grooming measured strongly correlated with each other across habituation days. On test day 1, a significant correlation between self-grooming and stereotypic episodes was observed, $r = .528$, $p < .001$, and for stereotypy count and self-grooming there was a strong negative correlation, $r = -.624$, $p < .001$. Stereotypy variables also negatively correlated with one another, $r = -.785$, $p < .001$ (Table 6). On test day 2, Self-grooming correlated with stereotypic episodes at $r = .435$, $p < .001$, and

self-grooming correlated with stereotypy count at $r = -.396$, $p = .004$. Both stereotypy variables correlated with one another at $r = -.785$, $p < .001$ (Table 7). On test day 3, self-grooming strongly correlated with stereotypic episodes, $r = .479$, $p < .001$, and with stereotypy count, $r = -.676$, $p < .001$. Stereotypy variables negatively correlated with one another at $r = -796$, $p < .001$. Therefore, we can consider grooming as a stereotypic behaviour.

Table 3. Correlation matrix for odour investigation variables (entries into the odour quadrant, approach towards the odour stimulus, and frequency of sniffing) on test 1.

Correlations

			1	2	3
1.	Odour Entries	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	Freq Approach	Pearson Correlation	.712**	--	
		Sig. (2-tailed)	.000		
3.	Freq Sniff	Pearson Correlation	.381**	.454**	--
		Sig. (2-tailed)	.006	.001	--

Table 4. Correlation matrix for odour investigation variables (entries into the odour quadrant, approach towards the odour stimulus, and frequency of sniffing) on test day 2.

Correlations

			1	2	3
1.	Odour Entries	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	Freq Approach	Pearson Correlation	.818**	--	
		Sig. (2-tailed)	.000		
3.	Freq Sniff	Pearson Correlation	.340*	.534**	--
		Sig. (2-tailed)	.015	.000	--

Table 5. Correlation matrix for odour investigation variables (entries into the odour quadrant, approach towards the odour stimulus, and frequency of sniffing) on test day 3.

Correlations

			1	2	3
1.	Odour Entries	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	Freq Approach	Pearson Correlation	.759**	--	
		Sig. (2-tailed)	.000		
3.	Freq Sniff	Pearson Correlation	.332*	.309*	--
		Sig. (2-tailed)	.017	.028	--

Table 6. Correlation matrix for repetitive behaviour variables (stereotypy count, stereotypic episodes, and self-grooming) on test day 1.

			1	2	3
1.	Self-groom	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	TotalSTREP	Pearson Correlation	.528**	--	
		Sig. (2-tailed)	.000	--	
3.	TotalSTRCNT	Pearson Correlation	-.624**	-.785**	--
		Sig. (2-tailed)	.000	.000	--

Table 7. Correlation matrix for repetitive behaviour variables (stereotypy count, stereotypic episodes, and self-grooming) on test day 2.

Correlations

			1	2	3
1.	Self-groom	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	TotalSTREP	Pearson Correlation	.435**	--	
		Sig. (2-tailed)	.001	--	
3.	TotalSTRCNT	Pearson Correlation	-.396**	-.790**	--
		Sig. (2-tailed)	.004	.000	--

Table 8. Correlation matrix for repetitive behaviour variables (stereotypy count, stereotypic episodes, and self-grooming) on test day 3.

Correlations

			1	2	3
1.	Self-groom	Pearson Correlation	--		
		Sig. (2-tailed)	--		
2.	TotalSTREP	Pearson Correlation	.479**	--	
		Sig. (2-tailed)	.000	--	
3.	TotalSTRCNT	Pearson Correlation	-.676**	-.796**	--
		Sig. (2-tailed)	.000	.000	--

3.6 Body temperature and body weight

The average change in peripheral body temperature post-injection of PPA and PBS increased for both groups across pre-loading days. The ANOVA revealed no significant main effect of Drug, $F(1,50) = .476$, $p = .494$. PPA treated rats had no significant difference in body temperature change compared to the PBS treated rats, therefore PPA did not significantly alter body temperature (Figure 10). Similarly, mean body weight steadily increased throughout the experiment for both PBS and PPA groups (Figure 11). The ANOVA revealed no effect of Drug, suggesting that PPA did not significantly impact body weight in comparison to the PBS groups, $F(1,49) = 2.616$, $p = .112$.

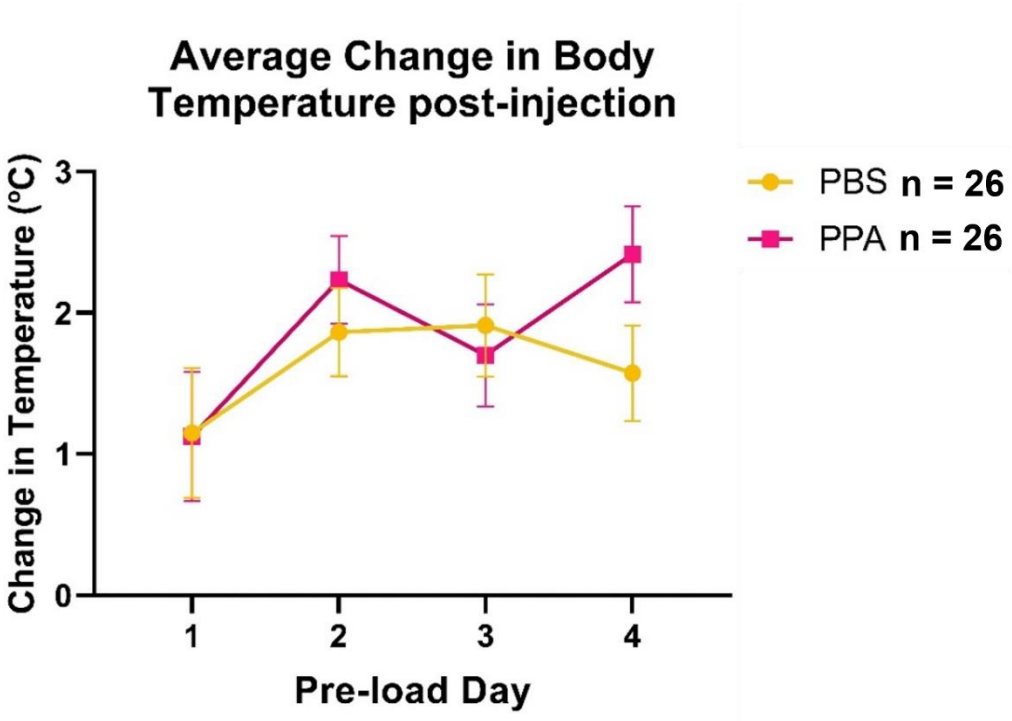


Figure 10. Average change in peripheral body temperature. The drug administered had no significant effects on body temperature. Data represented as mean ± SEM.

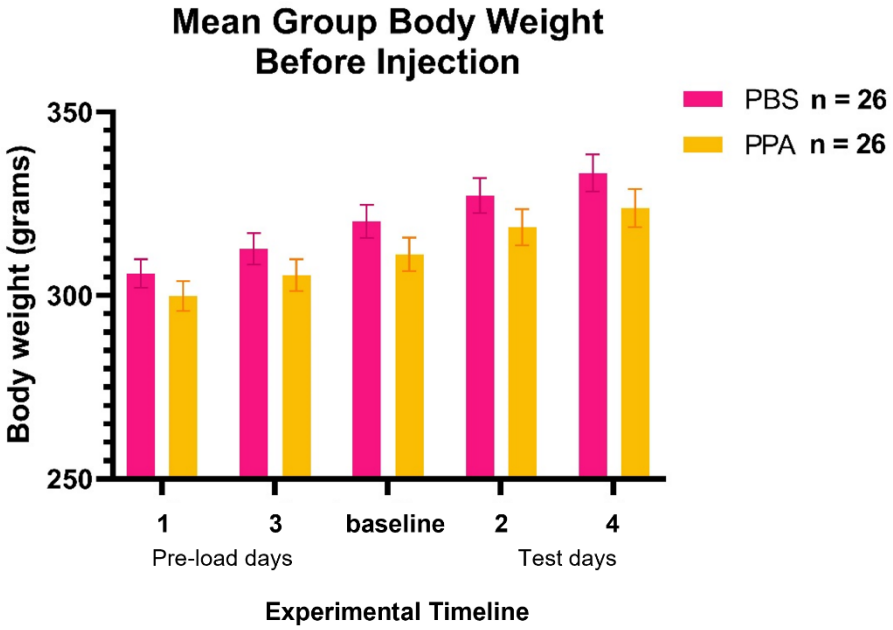


Figure 11. Mean group body weight before injection. Body weight steadily increased, and the drug administered had no significant effect on body weight across the experimental timeline. Data represented as mean ± SEM.

4.0 Discussion

The present study examined the effects of the short chain fatty acid PPA on odour habituation and locomotor activity in adult male rats. It was hypothesized that PPA treated rats would display signs of decreased social recognition and lack proper habituation and dishabituation to social odour cues. PPA rats exposed to non-social odours were predicted to habituate and dishabituate to the odours similar to PBS treated rats. The hypothesis was not supported, as repeated systemic administration of PPA did not significantly reduced odour habituation, however, for rats exposed to non-social odours, there was a trend of reduced habituation. The behavioural analysis showed that PPA non-social rats showed a lack of habituation across test days when travelling about the open-field, sniffing the odour cues, and entering the odour quadrant, however results were not significant. PPA groups also showed trends of reduced dishabituation for some exploratory and odour related measures, though these trends were not significant. Automated variables used in behavioural scoring strongly correlated with manually scored variables, suggesting that the Versamax software in the open-field is a reliable and valid method to score repetitive behaviours, and odour investigative behaviour. It was also observed that PPA treated showed reduced locomotion and increased repetitive behaviours in comparison to the PBS vehicle rats, which was consistent with previous literature.

4.1 Odour habituation/dishabituation task

The present study was designed to observe the effects of systemic PPA on social habituation by looking at the differences between PBS and PPA treated rats

in the retention of social odour-based memory. Ethologically relevant social odours and novel non-social odours were used to assess olfactory habituation and whether PPA had effects specific to social stimuli. In an altered version of the habituation/dishabituation task, rats are exposed to odours on cotton swabs 3 times for 2 minutes each with a short retention interval, then exposed to a novel odour on a different cotton swab in the home cage (Arubuckle et al., 2015). The sniffing time is recorded to measure olfactory responsiveness, or odour interest, where rats are expected to sniff for less time upon repeated exposures (habituation), and sniff for longer periods when a novel odour is presented (dishabituation). The odour test for the present study was an adapted version of the odour habituation/dishabituation task that occurred in the open-field across multiple test days (24h retention intervals), and had odours presented in a petri dish. Social odours were used as opposed to live conspecifics as previous research has indicated that soiled bedding can be a successful replacement as stimuli in social recognition tests (Sawyer et al., 1984; Burman & Mendl, 2002). Burman & Mendel (2002) used a similar method with soiled bedding encased in wire mesh as the odour stimulus. Both social and non-social odours were more potent and accessible in a petri dish on the floor of the chamber than on a cotton swab. Additionally, performing the task in the open-field allowed for multiple variables to be measured such as locomotor activity, self-grooming, approach towards the stimulus, and sniffing. Another benefit of using the open-field is that it eliminates the experimenter effect. In the original task, an experimenter must interrupt, and remove and replace the cotton swab, which may affect the results. Having large retention intervals between odour exposures also allowed for insight into PPA's

affects on long-term olfactory recognition memory. There may be limitations to 24h retention intervals as research suggests that odour recognition memory decays across time, however Feinberg et al (2012) showed that with only 1-minute exposures to either social or non-social odours in a study phase, vehicle rats were able to show novel odour preference in the test phase within retention intervals of 24 and 48 hours. However, strength and specificity of odour memory fades a function of time (Hackett et al., 2015). PPA's effect on habituation should be further explored by looking at various shorter retention intervals for exposure to social and non-social odours.

The PBS group failed to show normal responses to the odours, as the frequency of sniffing did not significantly decrease across repeated exposure days and did not significantly increase on the dishabituation day when the novel odour was introduced. This may be explained by another confound to the method used in this study, which was the change in location of the odour stimulus across test days, as counterbalancing the odours may have affected habituation. This is unlikely as Burman et al (2002) showed that conspecific odour recognition does not show context specificity. In a social odour recognition task, they used context-same and context-different (but similar environment) conditions, and rats showed novelty preference for both context conditions (Burman et al., 2002). In light of the differences in experimental procedures between the present study and that of Burman et al (2002), further investigation of the effects of changes in context on social/non-social odor habituation/ dishabituation are required in both the control (PBS) and PPA treated animals.

4.2 Locomotor activity

Locomotor activity measures including vertical activity, horizontal activity, and total distance travelled had significant drug effects where PPA induced hypoactivity 30 minutes after treatment (Figure 4). Other studies with systemically treated PPA rats have displayed hypoactivity in the open-field 60 minutes after treatment (Lobzhanidze et al., 2020). These results of reduced locomotion are consistent with previous results using a systemic PPA treatment (Ossenkopp et al., 2012; Shams et al., 2019; Wah et al., 2019). Shams et al. (2019) treated rats with PPA i.p. for four consecutive days, then followed by a 3-day break injected them again for 3 days while testing for locomotor activity in the open-field. Wah et al (2019) also observed hypoactivity in adolescence and adulthood from i.p PPA injections. Rats were injected twice in adolescence 3 days apart, and twice in adulthood 3 days apart, and both times the systemic PPA treated rats displayed decreased locomotion. However, previous studies that used direct central infusions of PPA via the ICV route found contradictory results, as rats experienced hyperactivity (McFabe et al., 2007; McFabe et al., 2008). The difference in results between these studies may be due to the differences in mode of administration, and the injection and testing schedule. In the ICV studies, rats were infused twice daily over seven days with 4µl of 0.26M PPA, and behavioural testing was performed after the second infusion (McFabe et al. 2007; 2008). In the present study, rats did not begin behavioural testing right away. Instead, they were pre-loaded with a systemic dose of 500mg/kg PPA for four consecutive days to allow PPA to exert its effects prior to behavioural experiments, and better represent pre-existing excess of PPA, similar to individuals with ASD. It can be

hypothesized that PPA may elicit changes in locomotion differently across time, where hyperactive effects are seen early on, and hypoactive effects are seen after longer periods of exposure.

Different routes of administration may result in different concentrations of PPA. Peripherally injected PPA must cross the blood brain barrier to gain access to the brain, where permeability to the weak acid may vary, whereas PPA infused into the ventricles bypasses this barrier and has direct entry into the brain. This is evident, as Cifuentes et al (2011) tested i.p versus ICV injections of the brain cell proliferation marker, bromodeoxyuridine, in rats, and showed that the ICV route had significantly better availability as more nuclei were labelled than in the i.p condition. Even with the same dosages, PPA concentrations in the brain are not the same following an ICV injection in comparison to an i.p injection, which may explain the difference in locomotor effects. Hypoactivity may also contribute to a decrease in odour investigation as the rat will approach and sniff the odour less often.

4.2.1 Exploratory behaviour

In the open-field, rats demonstrate inter-session habituation by decreasing their exploratory behaviour across repeated exposures to the novel environment (Leussis & Bolivar, 2006). Novel environments stimulate exploratory behaviour in animals, as they collect information related to factors related to survival such as potential food sources, mate availability, the presence predators, and possible escape routes (Leussis & Bolivar, 2006). After repeated exposure, the

environment becomes more familiar and habituation occurs as the need to explore decreases (Leussis & Bolivar, 2006). This is an indicator of learning and memory as the rat's behaviour is based on the recall/retention of their previous experience(s) in the environment (Leussis & Bolivar, 2006). Most of the PPA and PBS treated rats demonstrated habituation to the environment as measured by variables related to exploratory behaviour such as total distance travelled and entries into the odour quadrant. Figure 4 b highlights total distance and shows that test day 1 and 3 were significantly different from one another. This suggests that as a collective mean, the rats habituated to the novel environment. All groups experienced a decrease in total distance travelled by the 3rd test day, except for the PPA non-social group, that showed an increase in distance travelled compared to the 1st and 2nd test day. This may suggest that PPA treated rats failed to retain information about the novel environment (Leussis & Bolivar, 2006), and that PPA may interfere with odour recognition. Although there was no habituation in the PPA non-social group for total distance travelled, the PPA social group showed habituation, therefore the affect of PPA on habituation to a novel environment cannot be concluded from this. Looking at the 4th test day when the novel odour was introduced, there was no significant increase in exploratory behaviour for all groups, as test days 3 and 4 were not significantly different from one another. There was a minor rise in exploration of the open-field for PBS groups concerning total distance travelled and total horizontal activity (Figure 4), however, for both PPA groups, total distance travelled, and total horizontal activity decreased. These group differences were not significant, suggesting PPA had no significant effects on dishabituation to the open-field environment. PPA could potentially affect

odour discrimination in general, as PPA treated rats did not demonstrate the ability to detect novelty in the open-field, for both social and non-social odour stimuli. However, it is important to note that dishabituation did not occur for all groups, and although minor, group differences were not significant. The lack of dishabituation may have been due to the changes in context, as odour placement was switched between quadrants each experimental day.

4.3 Frequency of sniffing

All rats were able to detect the social and non-social odours, as they approached the odour stimulus and entered the odour quadrant multiple times throughout the experiment. Therefore, it is assumed that odour detection is not affected by PPA. However, there were differences in the frequency of sniffing for different odour exposures, and some minor differences in habituation and dishabituation between groups, suggesting that PPA affects odour discrimination. Both PPA and PBS treated animals sniffed the social odour significantly more than the non-social odour (Figure 7 a). This may be due to social odours being a more relevant stimulus as they are ethologically relevant to rats, unlike odours such as vanilla and almond extract that are non-food and non-social odours (Feinberg et al., 2012). It was unexpected to find that PPA social rats sniffed the odour stimulus significantly more than PBS and PPA non-social groups, considering various research highlights reduced social interest to unfamiliar conspecifics following PPA treatment (Shams et al., 2019; Choi et al., 2018). However, sniffing did not translate into enhanced approach and interaction, but it may relate to decreased habituation. Increased sniffing in the PPA social group

may have been due to the lack of physical and visual contact with the conspecific, which created a “socially safe” environment that motivated the rats to investigate the social odour (Tarland & Brosda, 2018).

4.4 Odour habituation

The rats displayed habituation to the odour stimulus as the overall sniffing frequency decreased across repeated exposures. This pattern of habituation was not observed in both the PBS non-social group and the PPA non-social group from test days 2 to 3. For the PBS non-social group, sniffing decreased from test day 1 to 3, however from test days 2 to 3 there was little to no difference in sniffing (Figure 7 a). For the PPA non-social group, rats decreased sniffing from test day 1 to 2, and increased sniffing on the last exposure day (test day 3) for the familiar odour (Figure 7 a). This increase in sniffing suggests a lack of habituation, however the difference in sniffing was not significantly different from the previous test day. The PPA non-social group also did not display dishabituation, which occurs when the physiological and behavioural response to a novel stimulus is enhanced for animals previously repeatedly exposed to a different stimulus (Steiner et al., 2014). When the novel odour was introduced on the fourth test day, the PPA non-social group was the only group with a decrease in sniffing frequency compared to the previous test day with the familiar odour (Figure 7 a), however this decrease in sniffing was minor, as there were no significant differences in sniffing across test days 3 and 4. For all other groups, there was an increase in sniffing on the 4th test day, however it was very minor. The difference in sniffing frequency on the 4th test day compared to the 3rd test

day was also not significantly different between groups (Figure 7 b), suggesting dishabituation to the odours was minor.

It is important to note that social and non-social odours are processed differently in the rodent olfactory system due to their unique compositions (Martinez-Marcos, 2009; Sanchez-Andrade & Kendrick, 2009; Feinberg et al., 2012). The two rodent odour processing pathways include the main olfactory pathway that typically detects volatile stimuli and the vomeronasal, or the accessory pathway, that responds to non-volatile and biologically relevant stimuli via direct contact (Martinez-Marcos, 2009). Since social odours can be comprised of both volatile and non-volatile components such as pheromones, excrement, and information regarding health status, age, sex, and relatedness, they are transmitted through both pathways. In comparison, non-social/ non-ethologically relevant odours are transmitted through the main olfactory pathway (Martinez-Marcos, 2009). In this experiment, volatile odours were used. Perhaps the lack of dishabituation when sniffing the novel non-social odour and the lack of habituation for non-social odour cues could be a result of PPA causing interruption to the main pathway of the rodent olfactory system. Another study that looked at social olfactory recognition examined nest seeking behaviour in rats prenatally exposed to PPA. PPA treated female and male pups showed an increase in latency to reach home bedding (Foley et al., 2014). This suggested PPA's potential role in impairing olfactory sensory processing and social recognition.

Although ASD is characterized by sensory functioning deficits in auditory, visual, and tactile systems, humans with ASD have also displayed olfactory impairment. Koehler et al. (2018) highlighted the atypical olfactory changes in those with ASD using a 2-phase study, starting with odour threshold detection and odour identification followed by structural and functional MRI tests. Phase 1 results showed that ASD participants had a reduced odour detection threshold, and reduced odour identification (Koehler et al., 2018). MRI results showed reduced activity in the piriform cortex, suggesting that olfactory deficits may be embedded in the primary olfactory complex (Koehler et al., 2018). Another study compared olfactory function in those with ASD and those with sensory processing dysfunction. Sweigert et al. (2020) found that ASD participants had intact odour detection but decreased odour identification ability that was significantly correlated with severity of ASD symptoms. The participants with sensory processing dysfunction showed deficits in both odour identification and detection ability (Sweigert et al., 2020). For habituation to occur, the animal must identify the odour/stimulus as familiar, therefore, if there is an olfactory deficit in ASD and those with sensory processing dysfunction, it will also affect their ability to habituate to odours. Although both ASD and sensory processing dysfunction groups showed olfactory issues, results suggest that differential patterns of odour processing are responsible for the primary olfactory deficits in ASD and in sensory processing dysfunction (Sweigert et al., 2020). Olfactory abnormalities and deficits are understudied in ASD and should be explored more to fully understand the sensory deficits associated with the disorder and the etiology behind these deficits.

4.5 Self-grooming, anxiety-related behaviour, and repetitive behaviour

PPA treated rats displayed a significantly higher rate of self-grooming than PBS groups across baseline and test days (Figure 8). This is inconsistent with previous PPA research that showed a decrease in self-grooming in rats systemically treated with PPA (Shams et al., 2019). This may be a result of methodological differences as Shams et al. (2019) used adolescent rats that were paired in the open-field with a live conspecific in treatment mixed and treatment matched conditions (PPA-PBS partners or PPA-PPA partners). Self grooms were affected by the presence of a conspecific, and non-volatile odours, and should not be a direct comparison to the present study that used volatile odours. Shams et al (2019) also began behavioural testing directly after injections, and in this study, behavioural testing began 30 minutes after injections to wait for peripheral PPA reaches the brain. Age is another factor to consider, as developing rats have greater blood-brain-barrier permeability to PPA than adult rats (Brusque et al., 1999). Age also impacts anxiety-like behaviours in the open-field, where adolescents display more anxious behaviour (Bishnoi, Ossenkopp & Kavaliers, 2020), however interestingly, anxious or stressed rodents should theoretically display more self-grooming (van Erp et al., 1994; Kalueff & Tuohimaa, 2005).

Although self-grooming is commonly performed as a self-cleaning behaviour, it can also act as an affective measure of anxiety as rodent grooming is sensitive to anxiety and stress (van Erp et al., 1994; Kalueff & Tuohimaa, 2005; Zhang et al., 2019; Estanislau et al., 2019; Rojas-Carvajal & Brenes, 2020). Rodent grooming

has a patterned organization, which includes nose/face/head wash, body wash, leg licking, and genital licking along with paw and tail licking (Zhang et al., 2019). Spontaneous grooming behaviour can occur in low stress conditions and typically involves a cephalocaudal pattern of licking, however more anxiety-like grooming includes disorganized patterning, interruptions, frequent switching between licking regions, and increased grooming frequency (Zhang et al., 2019). Self-grooming in this study was measured by the number of times rats switched between head washing, body/genital washing, paw licking, and tail licking, for an accurate measure of anxiety related behaviour. Other research suggests that self-grooming may act as anxiety relief, as part of a de-arousal process (Díaz-Morán et al., 2014; Estanislau et al., 2019). Anxiety is a large comorbidity of ASD, presumed to occur in 30 to 81% of young adults or children living with ASD (MacNeil et al. 2009; White et al. 2009). It is important to evaluate all aspects of the effects of PPA to find ways to potentially address the impact it may cause within ASD, or other neurological disorders.

Self-grooming is more often noted at as a measure of behavioural preservation, or repetitive behaviour modelling ASD. Repetitive behaviour is one of the main characterizing symptoms of the disorder (Arndt et al., 2005). The results showed that PPA groups displayed significantly more repetitive behaviour via self-grooming. Studies with ICV treatment of PPA in rats have also shown increases in repetitive and stereotypic behaviours (MacFabe et al., 2011; Meeking et al., 2020). Interestingly, repetitive behaviours in ASD have been associated with anxiety. ASD transgenic mice models have also shown increases in self-

grooming. The mouse strain BTBR T + tf/J models various behavioural phenotypes of ASD including reduced social behaviour and increased repetitive behaviours via significantly more self-grooming than the vehicle in adolescence and adulthood (McFarlane et al., 2008). Human studies show an increase of restricted and repetitive behaviours correlate with raised levels of anxiety in those with ASD (Tantam 2003; Rodgers et al., 2012). More research that supports the theory of de-arousal includes studies on children with highly restricted interests whose results suggest that restrictive and repetitive behaviours have an anxiolytic effect and may be a consequence of anxiety (Ooi et al., 2008; Spiker et al., 2011).

4.6 Automated versus manually scored variables

The manual and automated measures used in the current study strongly correlated with one another, suggesting that automated variables are sufficient at tracking repetitive movement and odour responses. In a study with mice, van dem Boom et al (2017) also compared automated and manually scored self-grooming in the open-field, as well as the elevated plus maze, using an open-source software (Janelia Automatic Animal Behavior Annotator). Using overhead video-recordings, the software was able to annotate bouts of self-grooming, and the total duration of a grooming session. van dem Boom (2017) and colleagues tested wildtype control mice and genetically modified mice with depletion of synapse-associated protein 90/postsynaptic density protein 95-associated protein 3in the striatum (SAPAP3 knockout mice), that have been observed to show increased self-grooming bouts and duration compared to controls. Their results revealed that the software detected significant increases in grooming behaviour in the SAPAP3

mice. Automated detection of total duration of grooming and number of bouts strongly correlated to the expert observer and was consistent with the observer 95% of the time when looking at duration, and 94% of the time when identifying bouts (van dem Boom et al., 2017). Sanberg et al (1987) showed that the automated Digiscan system, like the Versamax, used 16 infrared beams to track stereotypic behaviour (number of stereotypies and stereotypy time) in rats, and compared results to visual scoring, and saw significant correlations between the two methods. Using automated scoring methods is more efficient than video scoring as data can be gathered and analyzed quicker and assessed more accurately, as there is no inter-rater variability.

Automated scoring of rodent behaviour has also been proven reliable in multiple different paradigms. Rutten et al. (2008) showed that automated scoring of object recognition in rats is sufficient in examining memory performance during the object recognition test (ORT) as opposed to the usual method of manual scoring. They scored rat exploration time for small and large objects and concluded that correlations between automated and manually scored variables were substantially higher when smaller objects were using during testing, and that correlations were relatively low when larger objects were used, where the system showed overestimation in exploratory behaviour. The automated software was also able to sufficiently detect the difference in rat exploration time for treatment groups versus control groups in small object conditions, showing that automated scoring increases the validity and objectivity of the ORT (Rutten et al., 2008). Wahl et al (2022) used a novel automated approach to measure repetitive behaviours during

the marble burying test, in which rodents, commonly models of psychiatric and neurodevelopmental disorders, will bury varying numbers of marbles using the surrounding bedding in a cage. Normally, an experimenter will count the number of covered marbles at the end of the test, a method that is not standardized across laboratories, but with Wahl and colleagues' software, mouse behaviour was trackable during the experiment where information such as locomotor activity, number of and average duration of burying bouts and duration time of marble burying were monitored. Experiments performed using Ube3a $^{m-/p+,}$ mice models of Angelman syndrome, and wildtype mice showed that the automated system was able to detect significantly less burying behaviour in the genetically modified mice, which was consistent with existing literature (Wahl et al., 2022). To test reliability, manual video scoring was performed by 4 observers, whose results were similar to automated results, but showed inter-rater variability (Wahl et al., 2022), further showing the usage of automated software is sufficient at providing a replicable assessment of the marble burying test, and a superior method for standardization.

A common issue with manual scoring is that is liable to subjectivity and expectancy effects of the observer. Results of manual scoring will also vary across researchers. Automated variables are highly reliable as results are universal across software, and their use abolishes the possibility of subjectivity. Improving machine learning and creating software for automated scoring of animal behaviour will allow for more quantitative and accurate assessments, and in turn help progress our understanding mechanisms behind various behaviours.

4.7 Body temperature, body weight, and malaise

Reduced locomotor activity has been reported when rats are administered injections of the bacterial mimetic LPS, along with decreased body weight gain, and increased body temperature (Foley et al., 2014; Coelho et al., 1992). Since PPA is a bacterial product, there is a possibility that it may be able to induce malaise in a similar manner as LPS, and thus cause a decrease in locomotor activity via sickness response. Ossenkopp et al (2012) showed that i.p administration of PPA produced aversive internal cues, as PPA treated rats showed conditioned place avoidance, and conditioned taste avoidance. However, the present experiment showed that PPA had no significant effects on increasing body temperature compared to controls and did not affect body weight gain throughout the experiment (Figure 10 and Figure 11), therefore no sickness-like responses were observed. Previous studies that have used systemic PPA treatment also showed no signs of malaise, as PPA and PBS treated rodents had no significant differences in body weight gain after repeated injections (Shams et al., 2019). Wyse et al. (1998) treated rats twice daily with subcutaneous PPA for 15 days and weighed their brains at the end of the experiment and found that the weight was not significantly different from controls, meaning PPA does not cause malnutrition.

4.8 Potential mechanisms of PPA

Numerous explanations could potentially account for the behavioural effects of PPA found in this experiment and are mentioned below. To elicit

central effects, PPA passes through the gut blood and blood brain barriers via monocarboxylate transporters (O'Riordan et al., 2022). In the brain, PPA works on the peripheral neuron G-protein coupled receptors (GPCRs) GPCR41 and GPCR43 (O'Riordan et al., 2022) that are linked to intracellular calcium release (Le Poul et al., 2003). When an excess of calcium is released inside the cell, intracellular acidification occurs, which can lead to changes in neurotransmission, calcium signalling, and the inhibition of gap junctions (Karuri, Dobrowsky & Tannock, 1993). Excess calcium release can also lead to mitochondrial dysfunction (Yun & Lee, 2016). PPA disrupts SCFA oxidation, thereby inhibiting fatty acid metabolism (Brass & Beyerinck, 1988). Accumulation of PPA, or its metabolite, propionyl Coenzyme-A causes a decrease in acetyl-Coenzyme A and Coenzyme A, both critical for metabolism (Brass & Beyernick, 1988). Cannizzaro et al (2003) observed that intracellular acidification evoked dopamine release in rat hypothalamic synaptosomes, and dopamine mediates stereotypic behaviours in rodents (Moore & Grace, 2002), and may perhaps account for the increased grooming observed in PPA treated rats in this study.

4.8.1 PPA induces an inflammatory response

Histology reports from brain tissue of PPA treated rodents show that PPA induces a neuroinflammatory response, characterized by activated microglia that secrete cytokines (MacFabe et al., 2007). Human studies of ASD also report neuroinflammation and neuroglial activation in the white matter, cerebral cortex, and cerebellum of patients with ASD (Vargas et al., 2005). Estess and McAllister (2015) suggested that immune dysfunction may contribute to the development of

ASD, as multiple ASD associated genes encode elements of the immune system. Neuroinflammation may disrupt normal cognitive processes, as it also occurs in diseases like Alzheimer's and Parkinson’s disease. PPA has been implicated as a potential factor in the pathology of Alzheimer’s disease as it is present 1.35x more in the saliva of dementia patients than in healthy controls (Killingsworth et al., 2021). Salivary levels of PPA usually correspond to the cerebral spinal fluid (Killingsworth et al., 2021). Further evidence that supports this idea includes a study with fecal transplants from AD patients that were put into mice, as these mice had increased PPA levels compared to controls. (Killingsworth et al., 2021). If PPA is able to influence memory, this may explain why PPA treated rats in the present study showed signs of reduced long-term olfactory recognition memory. Neuroinflammation can also be induced by LPS and sickness, (Qin et al.,2007), however, immune components implicated in cognitive deficits are minimally evident in this study (refer to section 4.7).

4.8.2 Epigenetics

Initially, there are fast acting effects of PPA, however after repeated injections the mode of action of PPA's central effects may be through the modification of gene expression, or epigenetic actions. Like other SCFAs such as butyric acid, PPA is a histone deacetylase inhibitor, inducing phosphorylation of cyclic AMP response-element-binding protein (CREB) (Chen et al., 2003). This can trigger alterations in the expression of genes involved in learning and memory which may be behind symptoms of ASD.

4.8.2.1 PPA affects the expression of serotonin

Serotonin is a neurotransmitter which regulates multiple functions that can be affected in ASD. In the brain, serotonin modulates sleep-wake cycles, social behaviour, learning, pain sensitivity, appetite, aggression and impulsivity, and mood (Berger et al., 2009). In the periphery, where about 95% of serotonin is stored in the gut (Berger et al., 2009), serotonin modulates motor output, immune signaling, and other important physiological functions (Meredith et al., 2005). PPA is able to regulate the release of serotonin from enterochromaffin cells of the gut (Mitsui et al., 2005), which suggests that a potential mechanism by which locomotor activity is affected in PPA treated rats may be via PPA-mediated peripheral serotonin release.

Nankova et al. (2014) found evidence of PPA's role in the upregulation of peripheral serotonin. The rat pheochromocytoma cell line (PC12) was exposed to PPA, and they examined global changes in PPA-dependent gene expression in vitro and determined if these biological pathways were related to ASD. PPA was able to alter the serotonin system via tryptophan-5-hydroxylase 1 (TPH1) (Nankova et al., 2014). Research has found that that about one third of people with ASD have hyperserotonemia, or increased serotonin levels in the platelets (Melke et al., 2008; Mulder et al., 2004). PPA-induced increases in serotonin in the periphery may be related to motor output issues such as abnormal movements, hypoactivity and hyperactivity seen in ASD patients and PPA rodent models of ASD.

Peripheral levels of serotonin in people with ASD may be higher, however this does not represent an accurate amount of central serotonin, as gut-secreted serotonin cannot pass the blood brain barrier. TPH1 synthesizes non-neuronal serotonin, whereas TPH2 synthesizes neuronal serotonin, however they are both the rate limiting enzymes in serotonin biosynthesis (Nankova et al., 2014). In the brain, decreased serotonin has been implicated in ASD. Histological analysis of brain homogenates from PPA treated rats by El-Ansary et al. (2012) showed marked decreases in serotonin. In human studies with ASD patients, similar results were found that showed decreased central serotonergic activity (Azmitia et al., 2011). McDougle et al. (1996) found that decreased tryptophan, the precursor to serotonin, has been linked to repetitive behaviours in adults with ASD. These results are contradictory with other research, as fast acting mechanisms by which central PPA elicits behavioural changes may include pH dependent increases in serotonin via intracellular acidification (Meeking et al., 2020). Increases in central serotonin have also been linked to stereotypical and repetitive behaviours, and reduced social investigation in rodents (Langen et al., 2011; Gonzalez et al., 1996). Evidence of serotonin (5-HT)'s involvement in sociability includes a study with mandarin voles, where raphe nucleus 5-HT neurons were activated, and increased the release of serotonin during social grooming, sniffing, and social approaching of conspecifics (Li et al., 2021). Aberrations in serotonin expression can have drastic physiological and behavioural impacts and may be involved in the development of ASD or its symptomology.

Changes in locomotor behaviour may also be explained by 5-HT. Activation of 5-HT_2 G-protein coupled receptors has been implicated in affecting locomotor activity. Halberstadt et al. (2009) used mice to look at the effects of serotonergic hallucinogens that specifically act on 5-HT_{2A} and 5-HT_{2C} receptors and found that doses of the hallucinogen above 10 mg/kg decreased locomotor activity and doses around 1 mg/kg increased locomotor activity. In 5-HT_{2A} knockout mice, increased locomotor activity was blocked after exposure to the hallucinogen at low doses (Halberstadt et al., 2009). Similarly, decreased locomotor activity from higher doses was potentiated using 5-HT_{2A} knockout mice, suggesting that 5-HT_{2A} is involved in altering locomotor behaviour. The decreased locomotion was also potentiated by a 5-HT_2c agonist and blocked using a 5-HT_{2C} antagonist, suggesting that 5-HT_{2C} receptors mediate locomotor activity (Halberstadt et al., 2009). It is possible that these receptors could also be activated by PPA and exert effects on locomotor activity, however there is limited research to support this proposal.

5.0 Future Directions

In the present study, results may have shown stronger habituation in the PBS groups if there was no change in context and the odour cue remained in the same quadrant across the experiment. If the study were to be repeated, refraining from counterbalancing the petri dish may prove beneficial, and show significant odour habituation in PBS groups.

It would also be of interest to assess social recognition by the rat's ability to detect novelty by using an odour choice paradigm. Using the same 2-chambered open-field experimental design but adding two odour cues with one petri dish in each quadrant. The odours could be a familiar cage-mates soiled bedding on side and an unfamiliar rat's soiled bedding on the other. This way, if the rodent sniffs the unfamiliar stimulus more or is in the unfamiliar odour quadrant longer, it will show the rat is able to recognize that the social odour is unfamiliar, and if it sniffs the familiar odour more and stays in the familiar odour quadrant longer, it would suggest an impairment in social recognition, or odour discrimination.

Additionally, looking at the effects of other SCFAs should be considered in future studies. Similar SCFAs to PPA such as butyrate and acetate activate the same GPCRs (GPR43 and GPR41) and are also capable of modulating gene expression (Wang et al., 2012, McFabe et al., 2012; Nankova et al., 2014). In animals, when PPA is in excess, levels of butyrate and acetate will also be higher, as they arise from similar processes of fermentation by enteric bacteria (van der Hee & Wells, 2021). It would be of interest to see if there is a synergistic effect on behaviour from an excess of systemic butyrate, acetate and PPA together, or if PPA alone causes more behavioural deficits. ICV studies have already observed that PPA alone caused stronger behavioural changes in rats and more alterations in brain lipid composition than PPA and butyrate or butyrate alone (Thomas et al., 2010), however no study has compared peripheral effects of butyrate, acetate and PPA.

6.0 Conclusion

Research with peripheral injections of PPA highlights the gut-brain axis, and the ability of the gut microbiome to have significant neurological, physiological, and behavioural effects. Previous research has demonstrated systemic PPA's effects on decreasing locomotor and social activity, and the current study aimed to determine PPA's potential sensory effects, specifically on habituation and dishabituation to odours. In the present study, repeated exposure to PPA did not significantly affect the rats habituation to social odours, and showed a small trend of decreased habituation to non-social odours, however these effects were not significant. Further, PBS treated rats showed relatively little habituation across exposure days, and there was no evidence of habituation deficits in the PPA social group in comparison to the vehicle. PPA seems to have a general/non-specific effect on odour discrimination, as PPA treated rats failed to dishabituate to both social and non-social odour cues in the open-field, however dishabituation measures showed no significant results. The hypothesis that PPA treated rats would show impaired social recognition was not supported, as there were no significant changes in habituation or dishabituation in PPA groups compared to that of PBS. Results were also confounded by a lack of habituation/dishabituation in the PBS vehicle groups.

This experiment further validates the PPA rodent model of ASD as results of locomotor activity and repetitive behaviour were consistent with literature, and it was found that PPA may have additional effects on sensory mechanisms than initially thought. It was also observed that automatic variables are sufficient at

detecting odour investigation and repetitive movements, as visual scores for these variables strongly correlated to automated scores across test days. Moreover, it was found that systemic injections of the non-physiological dose of 500mg/kg of PPA does not induce malaise in rats, as body temperature nor body weight was significantly affected by the drug. Therefore, behavioural effects observed in rats from excess systemic PPA are due to a different underlying mechanism.

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Curriculum Vitae

Cashmeira-Dove Tyson

EDUCATION:

09/2020 – 08/2022 **Masters of Neuroscience (M.Sc.)**
University of Western Ontario
Thesis: The Effect of Propionic Acid on Habituation to Social and Non-social Odour cues in Adult Male Rats.
Co-supervised by Dr. Peter Ossenkopp and Dr. Martin Kavaliers

09/2016 – 04/2020 **Bachelor of Science (B.Sc., Honours), Biological Science**
University of Ontario Institute of Technology
Specialization: Life Science
Minor: Forensic Psychology
Capstone Thesis: The Effects of Rainbow Trout Diet on Plasma Metabolomic Biomarkers
Performed under the supervision of Dr. Denina Simmons

SKILLS:

Personal Skills

- Strong organizational skills, with the ability to prioritize and task manage
- Strong written communication skills developed through thesis projects, scientific report writing, and literature reviews.
- Confident articulate and professional speaking skills enhanced through participating in conference poster presentations, and seminar.
- Ability to teach and mentor undergraduate students.
- Efficient at strategic planning and conflict-resolution.
- Effective communication, interpersonal and relationship building skills
- Proficient in Microsoft Office applications and IBM SPSS Statistics
- Bilingual (B2 certified in French)

RELEVANT EMPLOYMENT:

01/2022 – 04/2022 **Teaching Assistant**

- Taught, reviewed, and ran in-class discussions concerning psychology as a social science (PSYCHOL 1003B) to first year undergraduate students. Ran weekly lab sessions via Zoom and in-class tutorials, and graded homework and discussion posts for four 10-30 student sections.

09/2021 – 12/2021 **Teaching Assistant**

- Taught, reviewed, and ran in-class discussions concerning psychology as a natural science (PSYCHOL 1002A) to first year undergraduate students. Ran weekly lab sessions via Zoom or in-

class tutorials, and graded homework and discussion posts for four 30-student sections.

09/2020 – 04/2021 **Teaching Assistant**

- Taught and reviewed research methods in psychology (PSYCHOL 2800E) in weekly lab sessions to second year undergraduate students. Ran labs teaching students how to use the free statistical software, JASP, provided office hours via zoom, and graded weekly homework, discussion posts, in-class assignments, as well as final projects. Provided support and guidance for students and tracked academic progress.
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05/2019 – 09/2019 **Research Assistant**
Dr. Denina Simmons Aquatic Toxicology; Aquatic Omics Lab, UOIT

- Responsibilities included external examination of fish, assisting with fish caging in local bodies of water, graphing, testing, and analyzing data, sample preparation for proteomics and metabolomics, and assisting graduate students with their Master's thesis projects.

02/2016 – 04/2016 **Respite Worker, Oshawa**
Looked after two children with muscular dystrophy and helped with their special needs by performing stretches and putting on leg braces. Also assisted with daily housework.

EXTRACURRICULAR INVOLVEMENT:

01/2022- 05/2022 **Science Rendezvous Executive, Communications Committee**
Social Media Manager, University of Western Ontario

- As social media manager, I organize cross-promotional posts with off-campus Science Rendezvous partners (e.g. Children's Museum); asks departments to advertise on their platforms; be in contact with Western Communications/Social Media; help with marketing materials.

11/2021- present **The Public Service Alliance of Canada (PSAC)**
Teaching Assistants and Postdoctoral Associates Union
Psychology Steward, University of Western Ontario

- As a steward, I act as the first point of contact for anyone (TA or postdoc) who needs help with a work-related concern.

12/2020- 04/2022 **Orientation & Social Committee (OSC), Society of Graduate Students**
University of Western Ontario
- As voting member of the OSC, helps to organize social events, and approve budgets and funding for various graduate clubs and societies at UWO.

10/2020- 10/2021 **Society of Graduate Students (SOGS) Neuroscience representative**
University of Western Ontario
- As part of the council, represents the neuroscience community within the largest graduate student advocacy organization and government at UWO. Engages in debate, and helps to provide services, benefits, and programs to ameliorate the grad school experience.

05/2018 – 04/2019 **Science Council, Vice President of Student Life**
University of Ontario Institute of Technology
- Represented the Science Faculty and aimed to better the quality of the student experience. Responsibilities included to create, run, and budget social events for the student body and to teach, mentor and delegate tasks to directors to run events smoothly.

02/2019– 05/2019 **Science Rendezvous Organizer, Marketing and Media**
University of Ontario Institute of Technology
- Collaborated with a student run team to create a fun, family-friendly, hands-on science community outreach event for all ages. Marketed the event across various social media platforms.

05/2018 **Science Rendezvous Volunteer**
University of Ontario Institute of Technology
- Interacted with youth and parents to demonstrate the significance of science in our society.

2018 – 2019 **Science Council Information Booth Volunteer**
University of Ontario Institute of Technology
Winter Open House
- Provided information to students about club events with peers, volunteering opportunities, campus facilities and

answered questions pertaining to science programs at the university.

2017 – 2018 **Biological Science Program Tour Guide/ Program Booth Volunteer**
University of Ontario Institute of Technology,
- Guided prospective students and parents throughout the university campus and answered questions pertaining to the program.

PROFESSIONAL COURSES:

- 11/2020 American College of Veterinary Surgeons (ACVS), hands on training
- 09/2020 Teaching Assistant Training program (TATP), Western University
- 04/2019 Canadian Council on Animal Care's Animal User Training Program
- 04/2019 Accessibility for Ontarians with Disabilities Act (AODA) Training
- 04/2019 Accessibility Standards for Customer Service Training
- 04/2019 Health and Safety Orientation for Workers
- 04/2019 Workplace Violence and Harassment Prevention
- 10/2018 Workplace Hazardous Materials Information System (WHMIS) Training
- 07/2018 OSSA (Ontario Science Student's Association) Executive training
- 09/2017 Lab Safety Training
- 05/2017 Smart Serve Responsible Service Training Program

RELEVANT POST SECONDARY COURSES:

University of Ontario Institute of Technology

Biochemistry Biochemistry I BIOL 2080U
Biochemistry II BIOL 3060U

Biology Biology I BIOL 1010U
Biology II 1020U
Cell Biology BIOL 2030U
Genetics and Molecular Biology BIOL 2020U
Human Anatomy BIOL 2050U
Introductory Physiology BIOL 2010U
Principles of Pharmacology and Toxicology BIOL 3020U
Developmental Biology BIOL 3051U

	Animal Physiology BIOL 3040U Fundamentals of Neuroscience BIOL 3060U Comparative Zoology BIOL 3610U Fundamentals of Nutrition BIOL 3650U Bioethics BIOL 4080U Neuropharmacology BIOL 4820U
Chemistry	Chemistry I CHEM 1010U Chemistry II CHEM 1020U Introduction to Organic Chemistry CHEM 2020U Organic Chemistry CHEM 2120U
Psychology	Introductory Psychology PSYC 1000U Developmental Psychology PSYC 2010U Abnormal Psychology PSYC 2030U Personality Psychology PSYC 3060U Forensic Psychology PSYC 3210U Eyewitness Psychology PSYC 3220U
Statistics	Statistics and Probability for Biological Science STAT 2020U

University of Western Ontario

Neuroscience	Perspectives in Neuroscience NEUROSCI 9500 Principles of Neuroscience NEUROSCI 9510
Psychology	Introduction to Statistics using R PSYCHOL 9041

SCHOLARSHIPS:

University of Ontario Institute of Technology

- 2016 Award of Recognition Entrance Scholarship
- 2019 Award of Recognition In-Course Scholarship

University of Western Ontario

- 2020 Graduate Research Scholarship in Neuroscience
- 2021 Graduate Research Scholarship in Neuroscience
- 2022 Graduate Research Scholarship in Neuroscience

HONOURS AND AWARDS:

- 09/2019– 12/2019 President's Honours List
- 01/2019– 04/2019 President's Honours List
- 09/2018– 12/2018 Dean's Honours List
- 2016 Ontario Scholar

PUBLICATIONS:

Kavaliers, M., Ossenkopp, K.-P., **Tyson, C.-D**., Bishnoi, I. R., & Choleris, E. (2022). Social Factors and the Neurobiology of Pathogen Avoidance. *Biology Letters* (2005), 18(2), 20210371–20210371. doi:10.1098/rsbl.2021.0371

CONFERENCE POSTER PRESENTATIONS:

Society for Neuroscience, November 11, 2021
Effect of Systemic Propionic Acid on Habituation to Odours and Locomotor Activity in Adult Male Rats: Modeling ASD.

Canadian Association of Neuroscience, May 14, 2022
Effect of the Bacterial Product, Propionic Acid, on Habituation to Social and Non-Social Odours by Adult Male Rats.

University of Toronto Collaborative Program in Neuroscience Research Day, May 27, 2022
The Effect of Propionate on Responses to Social and Non-Social Odour cues By Male Rats.