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Behavioral changes in a chronically administered Propionic Acid rat model of Autism Spectrum Disorders

A Thesis in Neuroscience

By

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Abstract

Spatial cognition, sociability and novelty preference, and anxiety-like behaviors were examined in a Propionic Acid (PPA) rat model of autism spectrum disorders (ASDs). Impact of sex and method of administration were of additional interest. We infused the drug over five days for chronic administration as opposed to acute administration to see if chronic exposure could produce similar behavioral changes. A first cohort of animals was given PPA through cannulas surgically implanted in the brain's intraventricular system. Acquisition and reversal training in the Morris Water Maze was conducted. No significant differences in search latency were found. Animals were tested for sociability impairments in a three-chamber sociability paradigm. No significant differences between PPA animals and controls were found. There was no impact of sex. Animals were run in an acoustic startle response test to assess anxiety-like behaviors. There was an effect of decibel and an effect of sex, such that males startled more. There was no effect of treatment. A second cohort of animals were given PPA intraperitoneally and run in the sociability protocol and acoustic startle response testing. No differences between treatment or sexes were found for sociability or acoustic startle. However, there was an effect of decibel and light on startle. Together, these results suggest that chronic administration of PPA may not produce the behavioral impairments in spatial cognition, sociability, and anxiety seen in previous literature employing acute administration. Implications of this are further discussed.

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INTRODUCTION

Autism Spectrum Disorders (ASDs)

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by social, cognitive, and locomotor deficits as well as perseverative and repetitive behaviors. This increasingly prevalent disorder affects an estimated 1/88 people; a 78% increase over 2002 prevalence rates (Martin & Horriat, 2012). ASD is an extremely variable, as of now incurable disorder that affects people of both sexes and all racial, ethnic, and socioeconomic groups (Valicenti-McDermott, et al., 2015).

Within the base categories of symptoms like social and locomotor, one finds striking potential for variance. Social deficits, for instance, may appear as atypical lack of social motivation in one individual while manifesting as inappropriately high levels of sociability. One child may be nonverbal, but capable of communicating in other forms while another can produce language, but often talks in repetitive fragments and echolalia. Some individuals are considered highly functioning and capable of living independently while others are severely afflicted and require lifelong care (Forsberg, Ilieva, & Michel, 2018). Comorbid conditions including epilepsy, physical abnormalities, and psychological disorders further complicate the presentation of autism. The extreme variability in symptom presentation is partially due to the DSM-V's diagnosis criteria for the disorder. Asperger's syndrome—once a separate entity characterized by "high functioning" individuals with social deficits—has been grouped with autism, childhood disintegrative disorder, and pervasive developmental disorders under the autism spectrum disorders umbrella (American Psychiatric Association, 2013). Thus, there is a

fundamentally greater likelihood of a child being diagnosed with an ASD. Autism may be referred to as autism spectrum disorder or pluralized--as it is here--to respect the fact that no two individuals on the spectrum are exactly alike in symptom presentation. No matter how one carves up the spectrum, however, significant variability is found within groups of similar profiles. This vast diversity in symptom presentation reflects a great swath of genetic and environmental factors that are thought to play parts in the disorders' etiologies.

Genetic and Environmental Factors

Autism is widely accepted as being highly genetic in its etiology as over 800 genes such as FOXP2, SHANK 2, and RELN have been implicated in the disorders (Barrett et al., 2017). Independent twin studies show that concordance rates for monozygotic twins are much higher (70-90%) than those of dizygotic twins (0-10%), supporting a strong genetic component in the etiology of autism (Chaste & Leboyer, 2012). Indeed, studies profiling families of afflicted individuals show that parents and siblings may be more likely to display cognitive and or behavioral features reminiscent of those observed in the broader autism phenotype (Abrahams & Geschwind, 2008). It is thought that there are different mechanisms of genetic transmission of autism as the rate of de novo genetic mutations and presentation of subclinical autism symptoms in family members seem to depend on whether the affected individuals disorder was sporadic or inherited (Chaste & Leboyer, 2012). Further, some researchers believe the domains of autism symptomatology may even be separately heritable while others argue that all

behavioral impairments can be linked back to one underlying neurodevelopmental issue (Chaste & Leboyer, 2012).

The many genes suspected of playing a role in Autism may affect multiple neurobiological pathways. Genetic disorders that have symptom overlap with Autism, including Fragile-X syndrome and Rett syndrome, tend to be associated with affected synaptic plasticity (Chaste & Leboyer, 2012). So too do individual gene mutations. Genome screening has revealed copy number variations (CNVs) in genes responsible for synapse formation and maintenance. However, mutations to these genes also affects cellular proliferation, motility, neurogenesis, and other important pathways (Chaste & Leboyer, 2012). Further complicating the genetic makeup of ASDs, identical genetic mutations in different people may correspond with divergent phenotypes. Thus, two people with the same mutation at the same loci may have a different disorder profile, or even two different conditions. Indeed, identical rearrangements of the 16p11 allele have been associated both with individuals with Autism and individuals with schizophrenia (Chaste & Leboyer, 2012). Coupled with the fact that not all sets of twins exhibit Autism, these divergent phenotypes point toward contribution from environmental factors capable of modifying phenotypic expression of Autism.

A pregnant woman's exposure to certain environmental factors; certain plastics, PCBs, herbicides and pesticides, and tainted drinking water may contribute to the development of an ASD. Advanced paternal age, increased levels of testosterone in the womb, and traumatic events in the mother's life, such as a death in the family, are thought to add risk as well. Though the mechanisms by which trauma may contribute to

the development of ASDs requires further research to be understood (Martin & Horriat, 2012). Childhood diseases may also contribute. For example, measles may cause encephalitis, or, swelling of the brain. This swelling is thought to be a possible cause of autism as many children with ASDs have larger heads, a sign of neurological inflammation (Schultz et al., 2009). According to the Centers for Disease Control and Prevention (CDC, 2015), children with ASDs are about 3.5 times more likely to suffer chronic diarrhea or constipation than their typically developing peers. Other gastrointestinal difficulties such as intestinal inflammation and GERD have also been reported in significant numbers (Buie et al., 2010). In addition, a strong positive correlation between gastrointestinal symptom intensity and ASD symptom intensity been found, suggesting a link between GI problems and ASDs (Li & Zhou, 2016).

ASDs and the Microbiome

The supposed interplay of a litany of genetic and environmental factors in the etiology of ASDs complicates researchers' ability to discern methods of ameliorating the disorders. If a certain cause was discovered for a group of individuals with one subgroup of ASDs; say one with a particular pattern of genetic abnormalities and exposure to the same environmental risk factors, treatment for those individuals would likely be unable to or limited in helping others on different parts of the spectrum. On top of this, knocking out one gene in an animal model cannot demonstrate the full complexities of the disorders (Barrett et al., 2017). To find as suitable a model as possible, it may be prudent to focus on an environmental risk factor known to possess the ability to activate genetic

changes related to developing ASDs. This search may take us to the relationship between the gut, the brain, and ASDs.

The microbiota-gut-brain axis is a bidirectional communication system through which gut microbiota may influence brain development and behavioral phenotypes (Li & Zhou, 2016). This communication occurs via neuroendocrine and neuroimmune mechanisms involving the autonomic nervous system (ANS) and enteric nervous system (ENS). It can be illustrated through the impact of stress. For instance, prolonged exposure to stress can result in overactive autonomic inhibition of intestinal motor function and gut secretion, leading to the destruction of gut epithelium. This in turn alters microbiota composition and activity. Overactivity of the HPA-axis may lead to greater levels of circulating cortisol and proinflammatory cytokines leading to intestinal inflammation and compositional changes in the microbiota.

There is increasing evidence supporting the role of the microbiota-gut-brain axis in ASD etiology (Li & Zhou, 2016). It is thought that a bacterial infection or chronic antibiotic exposure may result in gut microbiota dysbiosis that leads to behavioral and neurological impairment indicative of ASDs. Exposure to certain antibiotics may cause overgrowth of antibiotic-resistant bacteria in the gut, such as *Clostridia*, which produces a short chain fatty acid (SFCA), Propionic Acid (PPA), as waste (Nankova et al., 2014). Children with regressive ASDs, particularly those experiencing early hospitalization, infection, and antibiotic exposure, have been shown to possess, on average, atypically high levels of these PPA-producing bacteria in their stool. PPA, by virtue of being a SCFA, is capable of accumulating in circulating blood and crossing the blood-brain

barriers, having direct impact on the brain. PPA, through oxidization, creates acylcarnitines: potential biomarkers of some ASDs, that cause mitochondrial dysfunction in the brain. Atypical mitochondrial function and resulting energy expenditure has been thought to be a possible factor in the development of ASDs. This is not, however, the only mechanism through which PPA may be involved in the etiology of ASDs.

PPA is structurally and pharmacologically similar to another SCFA, Valproic Acid (VPA) which is a known risk factor for ASDs and can be used to create a widely accepted rodent model of ASDs (Nankova et al., 2014). Both SCFAs, as well as other gut SCFAs, can act as histone deacetylase inhibitors, making them capable of altering the expression of ASDs related genes and activation pathways, thus contributing to the manifestation of the disorders. Aside from the gastrointestinal difficulties experienced by many individuals with ASDs, PPA has been tied to general developmental delay and seizure activity; two more common problems for people with ASDs. PPA infused rats have been shown to express a wide range of ASD-like symptoms (Schultz et al., 2009).

The PPA Rat Model of ASDs

The PPA Rat Model of ASDs is an established model of the disorders, producing neurochemical, physiological, and behavioral changes in rats indicative of human symptomatology (Nankova et al., 2014). ASD-like alterations in neurotransmitter levels, brain inflammation, sensorimotor difficulties, perseverative behaviors and seizure activity are a few salient features of the model. Schultz et al. (2009) revealed these symptoms through brain assays and behavioral tests after intracerebroventricular (ICV) infusions of PPA into rats. Their PPA animals performed within comparable levels to controls during

acquisition training in the Morris Water Maze. Interestingly, they demonstrated longer search latencies and a tendency to enter the quadrant of the original platform from acquisition training during reversal training. Typically, PPA rats show decreased latency in reversal training. These findings were attributed to perseveration of responding to the original platform quadrant because these rats also displayed significantly greater tendencies to swim for the original quadrant at the start of reversal training. This perseveration may be consistent with humans with ASDs who resist change and pursue repetitive interests.

This study also employed the beam task in which the animals traveled along a beam while locomotor performance was assessed. PPA animals displayed significantly more slips and falls than controls with four animals displaying convulsive behavior (Schultz et al., 2009). Accounting for the convulsive animals, this significant difference was maintained, implying sensorimotor impairment in the PPA group. This impairment in fine motor skills is reminiscent of humans with ASDs who may display stereotyped movements and gait disturbances. The neurobiological changes thought to be responsible for these behavioral deficits in PPA rats; neuroinflammation, oxidative stress, mitochondrial dysfunction, and altered serotonin levels, have also been found in humans with ASDs (Schultz et al., 2009).

Anxiety

Anxiety is a frequently comorbid condition for humans with ASDs with up to 80% of children with ASDs experiencing clinically significant levels of anxiety (Nadeau et al., 2014) and about 40% of individuals having at least one anxiety disorder

(Argyropoulos, Gilby, & Hill-Yardin, 2013). This anxiety, which may manifest in generalized anxiety disorder, obsessive-compulsive disorder, and separation anxiety disorder, as well as social phobias, has been linked to increased impairment in psychosocial, familial, behavioral, and academic aspects of functioning. Changes in gastrointestinal function related to ASDs are thought to be connected to anxiety, however, enteric mechanisms involved in such an interaction have not been thoroughly investigated (Argyropoulos et al., 2013).

The VPA rat model has demonstrated increased levels of anxiety-like behaviors, with rats spending more time in the "safer" outer zone than the inner zone of an open field. Various genetic mice models have also shown greater anxiety in open field tests of anxiety. The PPA model has been shown to produce increased anxiety related behaviors in open field tests and the elevated plus maze (Foley, Ossenkopp, Kavaliers, & MacFabe, 2014). The open field and elevated plus maze test run on the idea that rodents are predisposed to view open and unknown environments as dangerous. Thus, staying away from the potentially unsafe center of an open field or the open arm of the maze indicates greater anxiety-like behavior.

Another assay of anxiety-like behaviors in rats is the Acoustic Startle Response (ASR). Rats are presented with sudden bursts of noise at varying decibel levels and their ASR, or how much they physically startle in response to the sounds, is recorded as a measure of anxiety-like behavior. Typically, fear-potentiated ASR is used, in which a rat learns to associate a stimulus with a foot shock. This type of anxiety-like behavior is considered phasic. That is, it is largely time locked to the presence of a certain stimulus

and dissipates soon after. This implies that the generated ASR may be more indicative of transient fear states than pervasive anxiety.

Research shows that conditioned ASR does not respond to anxiolytic drugs, suggesting that it may not be a good model of anxiety in humans (Davis, Walker, Miles, & Grillon, 2010). Conversely, tests producing long-lasting states of apprehension, or sustained fear, do respond to anxiolytics. Research into phasic and sustained fear in rodents shows that, while both types of fear are mediated by the amygdala and send output to the hypothalamus and brainstem, sustained fear also causes the amygdala to release stress hormone which acts on receptors in the bed nucleus of the stria terminalis (BNST). Thus, despite the overlapping brain region activation and conceptual similarities between fear and anxiety, the sustained fear model activates the neurological pathway that can produce anxiety-like behavior more indicative of that in humans.

In the interest of devising a model for sustained fear in rats, Walker and Davis added another sensory element to the typical acoustic startle test by exposing their animals to bright lights (Davis et al., 2009). This Light-Enhanced Startle (LES) test exposes rats to sudden noise bursts at varying decibel levels in the dark and then the light, or in the dark again. This creates a sustained state of apprehension with less predictable threats and allows for comparison between startle response in darkness; which rats are more comfortable in, and bright lights; which rats naturally find aversive. They found that rats startled at greater intensities when exposed to bright lights following darkness. Walker & Davis (2001) further found that the anxiolytic, buspirone, did not lessen ASR, however, it did decrease light-enhanced ASR, suggesting this model of anxiety-like

behaviors may be more indicative of anxiety in humans. Humans, being diurnal as opposed to nocturnal, have been found to exhibit dark-enhanced startle that can be blocked by the anxiolytic, benzodiazepine diazepam (Davis et al., 2009). This seems to mirror LES in rats, further suggesting that it can create anxiety-like behaviors indicative of humans.

It is of interest to examine pervasive anxiety in a PPA rat model of ASDs because many people with ASDs suffer from chronic anxiety disorders and would benefit from better understanding of the nature of their anxiety, treatments, and potential relations between gut dysfunction and pervasive anxiety.

Social Functioning

One of the core symptom domains of ASDs is social functioning. Individuals with ASDs typically display social impairments in the form of reduced sociability and social motivation or inappropriate social interaction and initiation (Hazlett, 2016). Reduced social interaction was found in the VPA model of ASDs in a sociability and social preference test. VPA rats spent significantly less time with a familiar rat than an empty chamber and made more entries in to the empty chamber relative to controls (Kim et al., 2010). Because production of enteric SCFAs, like PPA, are linked to antisocial behavior in humans (MacFabe, 2009), it seems possible that a PPA rat model might also see decreased sociability. Indeed, acute ICV infusion of PPA in rats has been shown to increase mean distance PPA animals keep from each other in an open field, decrease time spent in close proximity, and reduce playful interactions (MacFabe et al., 2010). Another study investigating effects of PPA on social behavior looked at preference for objects

compared to novel rats. As seen in humans with ASDs, the PPA animals tended to prefer novel objects to novel conspecifics when able to choose between them (MacFabe et al., 2010). These studies show that the PPA model is able to create social impairments comparable to the human disorder.

One question that arises from these studies is: what would happen if a PPA animal could choose to interact with a familiar rat or a stranger, as opposed to an object? This question is of interest to our research because humans with ASDs have been shown to prefer familiar people over strangers, showing either neutral interest or even immense anxiety or fear of new people (Munson et al., 2006). A behavioral test that might probe such a question is Crawley's Sociability and Preference for Social Novelty Protocol. It has been helpful in assessing social impairment in genetic ASD rodent models (Kaidanovich-Belling et al., 2011) as well as the VPA rat model (Kim et al., 2010), but it has not been used in conjunction with the PPA model. This three-chamber paradigm allows researchers to measure animals' sociability and preference for novel strangers over old strangers by recording and comparing time spent with and distance from strangers. Other social behaviors can be tallied like sniffing, freezing, or self-grooming. This may be a useful tool for deeper exploration of the nature of social impairments brought about by the PPA model as it assesses general sociability and stranger novelty preference as well as other types of behaviors that belie comfort or discomfort around another animal. This test also allows for clearer determinations to be made about the experimental animal's social engagement and initiation of interactions as the control animal's movement is restricted.

Sex Differences

While some studies with PPA rodent models of ASDs have compared male and female cohorts or featured an entirely female cohort as opposed to an entirely male one, the vast majority are male dominated. This is likely due to the ease of using one sex, particularly males as they do not go through the estrus cycle, and the 3:1, male to female ratio of ASDs in humans (Strasser, 2016). Of the studies that have included females, sexually dimorphic differences for social dysfunction, anxiety, and other behaviors have been found (Strasser, 2016), warranting further examination of sex differences in PPA models of ASDs. Of particular relevance to the current study, sociability is often less impaired in female humans with ASDs. This is thought to be due to the protective effects of estrogen against the development of ASDs which may also contribute to the significantly smaller female population with the disorders (Strasser, 2016).

ASR testing provides further opportunity to assess sexually dimorphic anxiety in rats. Toufexis (2007) found that BNST-mediated sustained potentiated startle, but not fear-potentiated startle was increased in female rats compared to males. In a test of standard ASR, Foley et al. (2015) found that postnatal PPA treatment decreased startle in males, but increased startle in females. It would be of interest to determine whether light-enhanced ASR, which adds another sensory modality, creates the same changes in responsivity. The growing evidence of sexually dimorphic symptom presentation in human ASDs and the PPA model, calls for further research to tease apart these differences and better understand underlying neurobiological etiology to produce better treatment options for both sexes on the spectrum.

The Current Study

The Propionic Acid rat model of ASDs is considered a relatively robust animal model for studying the disorders and understanding possible gut-brain mechanisms behind the development of some forms of the disorders. The purpose of this study was to further explore certain aspects of this model's validity. While the PPA model's face validity, or ability to create behavioral impairments consistent with human ASDs (Tania, Khan, & Xia, 2014), is well supported by the literature, there are other aspects of ASDs that could be better understood through an animal model like the PPA model. Determining whether this model can produce these aspects can simultaneously bolster this model's face validity and be of use to further research in understanding the mechanisms behind them and developing treatments.

We looked at anxiety-like behavior in a light-enhanced acoustic startle test to see if this model can create anxiety-like behavior in response to acoustic and visual stimuli including loud sounds and bright lights respectively. We were also interested in expanding the literature on sexually dimorphic symptoms in human ASDs and the PPA model by looking for impacts of sex on sociability and anxiety measures. We also wanted to replicate previous findings of perseverative behavior in order to compare our model to previous literature.

It was hypothesized that PPA rats would exhibit perseverative behavior during reversal training in the MWM as shown by significantly longer search latencies than controls. A second hypothesis was that PPA animals would express significantly less sociability and preference for social novelty than controls with males showing

significantly less sociability and preferences for social novelty than females in a three-chamber social paradigm. It was also hypothesized that PPA rats would exhibit significantly increased levels of ASR and LES response. In addition, PPA males were anticipated to have decreased ASR compared to controls and PPA females were expected to have increased ASR compared to controls.

METHODS

Subjects

48 male and female Sprague-Dawley rats were used over the course of this study. All animals were allowed access to food and water ad libitum and kept under a 12:12 dark/light cycle. Treatment of animals was approved by an IACUC committee. Rats were run in two cohorts with two different experimental procedures.

Experiment One

PPA Administration

The first experimental group consisted of a total of 15 animals; eight experimental animals and seven controls. Based on previous research, this group received ICV cannula implantation surgery so that the drug could be delivered directly via cannula. Rats were deemed ready for surgery once they had surpassed 200 grams. Rats were mounted in a standard stereotaxic frame in aseptic conditions. Isoflurane was administered through a nose cone to maintain anesthesia throughout the surgery. The cranium was exposed so a 23-gauge guide cannula could be implanted in the right lateral ventricle. The tip of the

guide cannula was placed at the following coordinates with reference to Bregma: anterior/posterior −1.4 mm; medial/lateral 1.8 mm; dorsal/ventral −3.0 mm（Schultz et al, 2009). Four stainless steel screws were inserted into the skull and around the cannula so that dental acrylic could be applied, thus attaching the cannula to the skull. The guide cannula was sealed with a removable cap for the interim between surgery and infusion. All rats were given a one-week recovery period after surgery before infusion. 0.26 M PPA was buffered to 7.5 PH and delivered in 4 µL doses (Schultz et al., 2009) over 5 consecutive days to test for a chronic effect of the drug on behavior. Control animals received the same surgical and infusion procedures as experimental animals, while being infused with equivalent amounts of saline instead of drug. This experimental group was used in three behavioral tests; MWM, Crawley's Sociability Protocol, and Light-enhanced ASR.

Morris Water Maze

MWM protocol specified by Schultz et al. (2009) was used for the current study. Rats were placed gently into a circular pool of water facing a wall in a randomly chosen quadrant of the pool. They were left to swim to a platform hidden a couple inches below the water. If a rat failed to find the platform at 60 seconds, they were picked up by the experimenter and gently placed on the platform where they could sit for 15 seconds. Rats were toweled off carefully after each trial and placed in their cages. After 10 trials of acquisition training, rats were given a one-week recovery period. Following that, they ran through 10 trials of reversal training in which the protocol is identical to acquisition training, but the platform is in the opposite location. Latency to platform and time spent

in the zone containing the platform was recorded for all 20 trials for later analysis. Data and video was obtained through ANYmaze software (Wood Dale, IL, USA) for future statistical analysis.

Crawley's Sociability and Preference for Social Novelty Protocol

To test for social behavior impairments, Crawley's sociability and preference for social novelty protocol was employed (Kaidanovich-Belling et al., 2011). The protocol was carried out in an open field box with two, small identical cages on either side. The experimental rat was placed in the center zone of the box for a five-minute habituation period. After this period, a control rat was placed in one cage and the experimental rat free to roam about the box for ten minutes. Placement of the stranger rat into the left or right cage was alternated for each test to avoid confluence from possible side preferences. Average distance from the control rat and the empty cage were recorded. The same procedure was followed for a second ten-minute session with the addition of a novel rat in the other cage. Distances from each rat and or empty cage for each of the three sessions were recorded for later comparison and analysis. Data and video was obtained through ANYmaze software (Wood Dale, IL, USA).

Light-Enhanced Acoustic Startle Response

To test for increased anxiety-like behavior in rats, light-enhanced acoustic startle response was assessed (Davis et al., 2009). Rats were allowed to acclimate to their startle boxes for five minutes while background white noise of 65 to 68 dB played through a speaker. Acoustic startle amplitude was measured for two, 20-minute sessions. The first session occurred in the dark with 30 startle-eliciting noise bursts (ten bursts at 90, 95, and

105 dB each presented in an irregular order). The second session followed the same procedure; however, light was provided by a white fluorescent bulb (8 W). Amplitude of startle responses were recorded with software (MedAssociates, St. Albans, VT) for comparison between experimental groups and controls and further analysis.

Experiment Two

PPA Administration

Intraperitoneal (IP) injections deposit drugs into the peritoneum or body cavity. They are more expedient than ICV infusions because they do not require surgery and can be performed quickly and efficiently. IP administration is a valid tool for creating a PPA model because it has been shown to produce anxiety-like behaviors and social dysfunction in rats seen in human ASDs and comparable to behaviors of other animal models of the disorders (Strasser, 2016).

For our study, a second cohort of 20 animals; 10 experimental and 10 controls, were administered PPA directly through intraperitoneal injections. The experimental group was delivered the drug for five days at 2 mL per kg of rat of a 2.5 mg PPA/ml PBS solution (Foley et al., 2014). The control group was given saline with the same administration method and schedule. The same protocols from Experiment One were used to assess sociability and light-enhanced acoustic startle response in this cohort.

Statistical Analysis

Data was analyzed using the statistical package for the social sciences (SPSS, Chicago, IL, USA). Figures were created using Microsoft Excel or generated from ANY-maze software (Wood Dale, IL, USA). To determine effects of treatment and sex on dependent variables, data values were averaged and one-way and two-way analysis of variance (ANOVA) were run. Significance was assigned at the level of $P < 0.05$.

RESULTS

Experiment One

Morris Water Maze

We examined search latency in MWM acquisition and reversal training sessions. Based on previous literature, we expected to find a significant interaction between trial and latency such that increasing trials would see decreasing search latency. We also hypothesized a significantly greater search latency for PPA animals overall, compared to controls. Interestingly, PPA animals seemed to start off with shorter latencies than controls, while ending up with longer latency for trials four and five for both trainings (See Figures 1 and 2). For reversal training, PPA animals actually ended up with an almost identical search latency to their latency for trial 1. From figures 1 and 2, there appears to be a dramatic decline in latency for control animals around trial three that PPA animals did not experience.

We ran a repeated measures ANOVA on search latency between treatment groups with trial as a repeated measure. For acquisition training, there was no main effect of treatment group on search latency ($F_1=1.305$, $p>0.05$), but there was a main effect of trial

on search latency (F_4=9.229, $p<0.05$). For reversal training, there was no main effect of treatment on search latency (F_1=0.388, $p>0.05$), but there was a main effect of trial on search latency (F_4=4.379, $p<0.05$). For both acquisition training and reversal training, there was no interaction of treatment and trial (F_4=1.463, $p>0.5$; F_4=1.717, $p>0.5$). Our analysis does not support our hypothesis that PPA animals would exhibit significantly longer search latencies overall. However, it does support our hypothesis that latency would decrease with increasing trials.

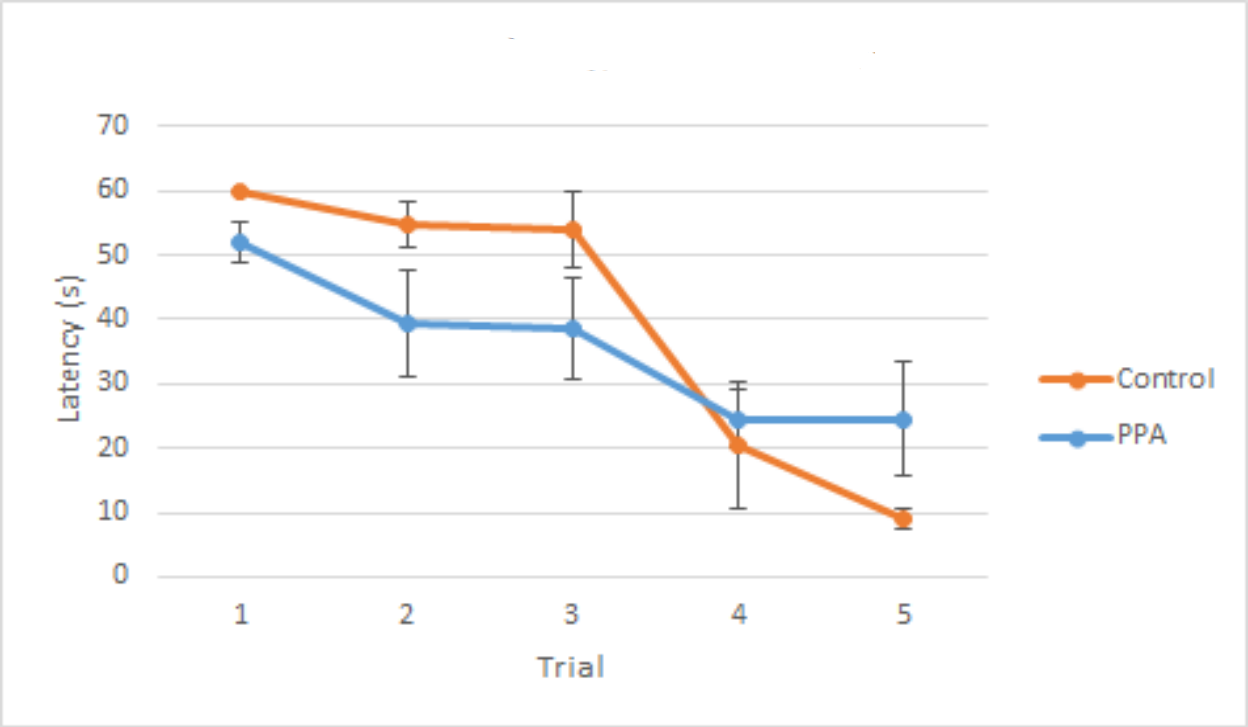

Figure 1: Acquisition Training Latency shows average search latency for platform during acquisition training across all five trials for PPA animals (n=8) and controls (n=7).

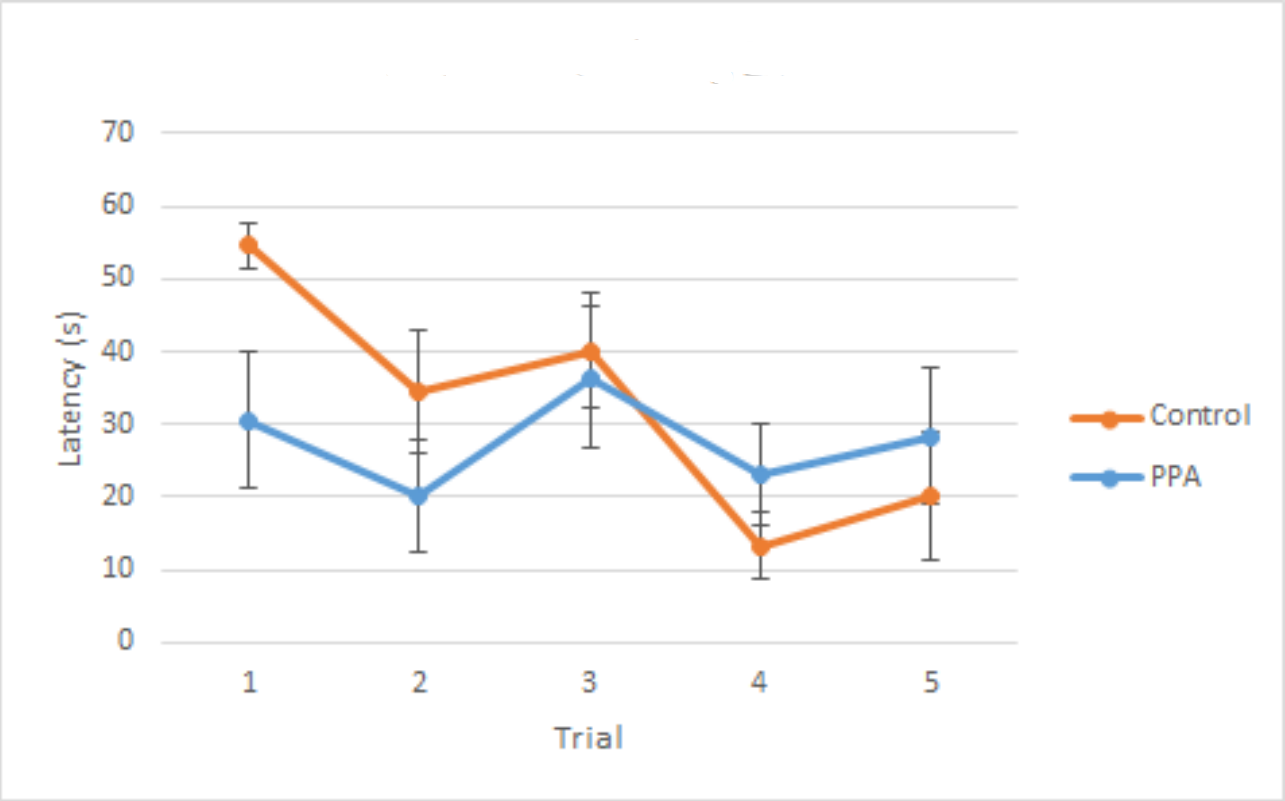

Figure 2: Reversal Training Latency shows average search latency for platform during reversal training across all five trials for PPA animals (n=8) and controls (n=7).

Crawley's Sociability Protocol

We examined sociability and novelty preference in Crawley's Sociability Protocol, anticipating that PPA animals would exhibit significantly lower levels of sociability and less preference for novel stranger compared to original stranger. We also hypothesized that males would display significantly less sociability and novelty preference than females in the PPA group. Broken down by social affiliation and social novelty stages of the test, average distances from the experimental rat to the stranger and the empty cage or novel rat were calculated for each treatment group and sex group.

First, we compared distance from rats/empty cage in PPA animals and controls during SA stage. There was a marginally significant difference between PPA animals (M=0.6, SD=0.1) and controls (M=0.5, SD=0.1) for empty cage; t(13)=1.975, p=0.070. There was no significant difference between PPA animals (0.4, SD=0.1) and controls (M=0.4, SD=0.1) for stranger rat; t(13)=-0.747, p=0.469. In the second independent samples t-test, we compared distance from rats/empty in females and males. There was no significant difference between females (M=0.6, SD=0.1) and males (M=0.5, SD=0.1) for empty cage; t(13)=.477 and no significant difference between females (M=0.4, SD=0.1) and males (M=0.4, SD=0.1) for stranger rat; t(13)=0.247, p=0.808.

For SN stage, we first compared distance from rats in PPA animals and controls. There was no significant difference between PPA animals (M=0.5, SD=0.1) and controls (M=0.5, SD=0.1) for stranger rat; t(13)=0.021, p=0.864. There was no significant difference between PPA animals (M=0.4, SD=0.1) and controls (M=0.4, SD=0.1) for novel stranger rat; t(13)=0.435, p=0.671. Next, we compared distance from rats in

females and males. There was no significant difference between females (M=0.6, SD=0.1) and males (M=0.4, SD=0.1) for stranger rat; $t(13)=1.524$, $p=0.152$. There was no significant difference between females (M=0.4, SD=0.1) and males (M=0.4, SD=0.1) for novel stranger rat; $t(13)=-0.869$, $p=0.286$.

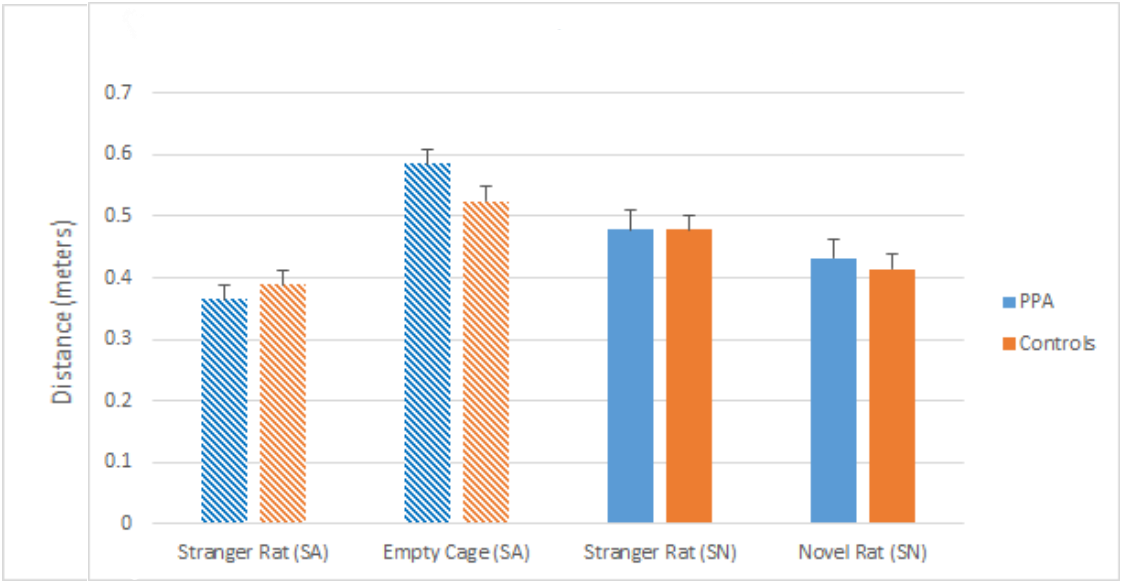

Figure 3: Average Distance from Rats shows the average distances in meters of experimental animals (n=8) and controls (n=7) from the stranger and empty cage or novel rat for each stage. SA stands for Social Affiliation stage and SN for Social Novelty stage.

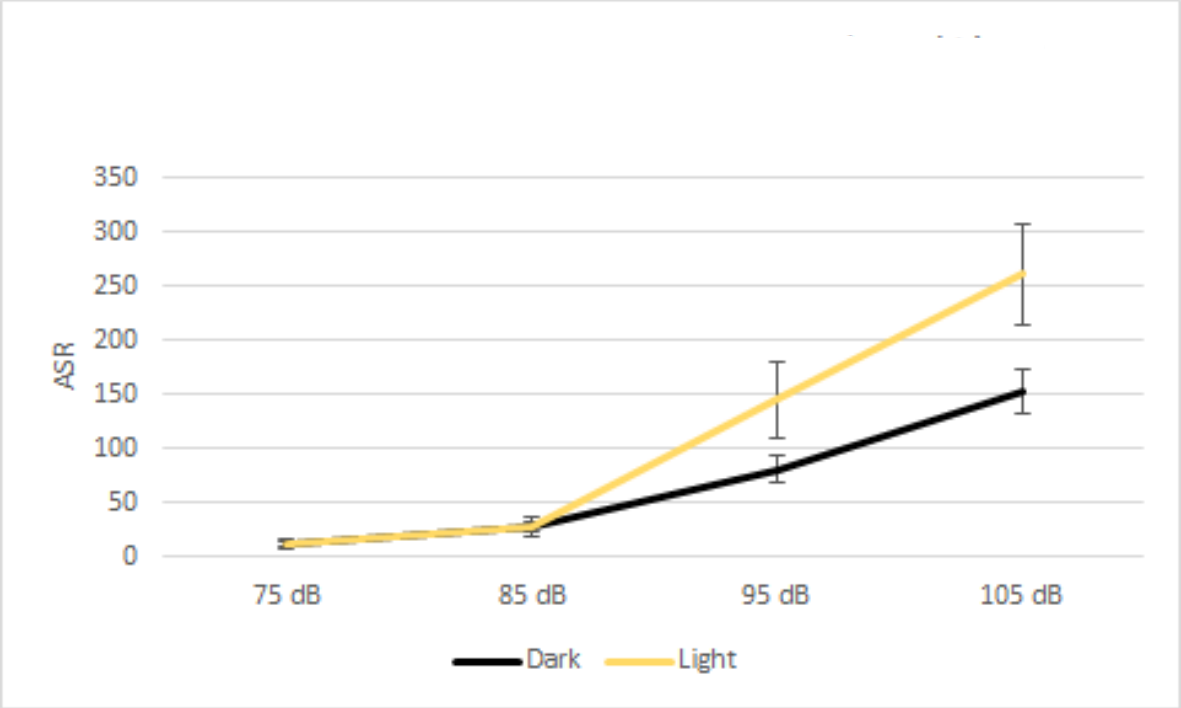

Figure 4: Average LES Response shows untreated control (n=8) data for LES response across four decibel levels.

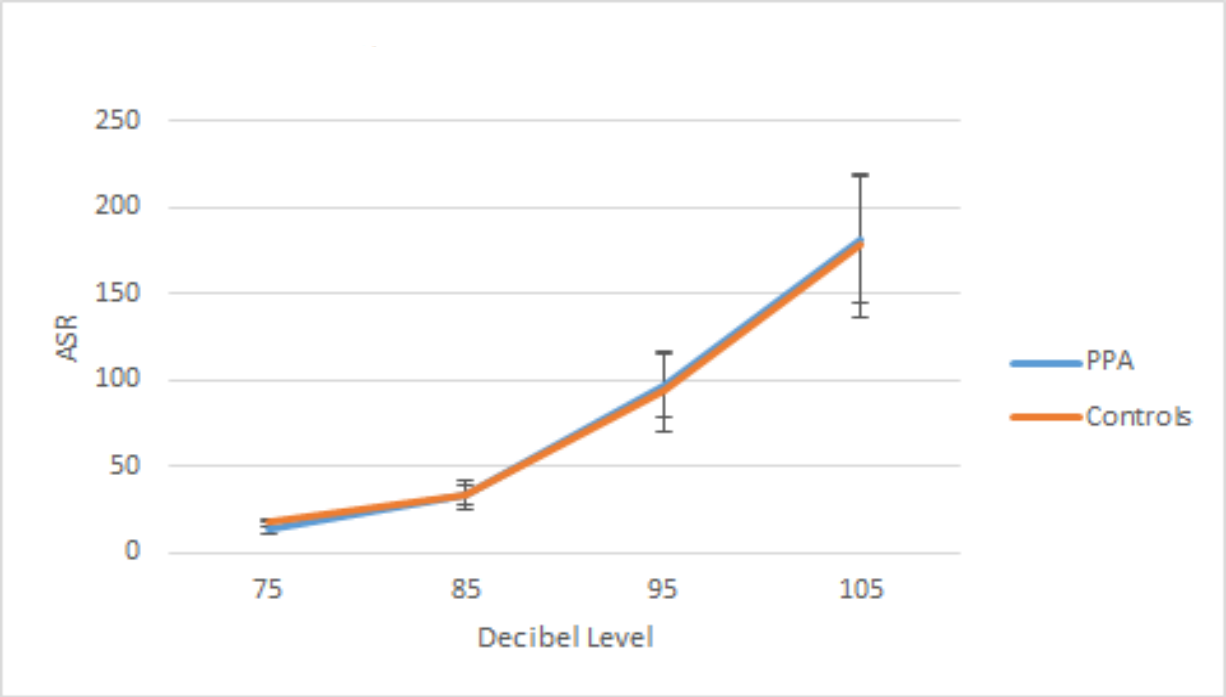

Figure 5: Average ASR by Decibel Level for PPA (n=7) and controls (n=8).

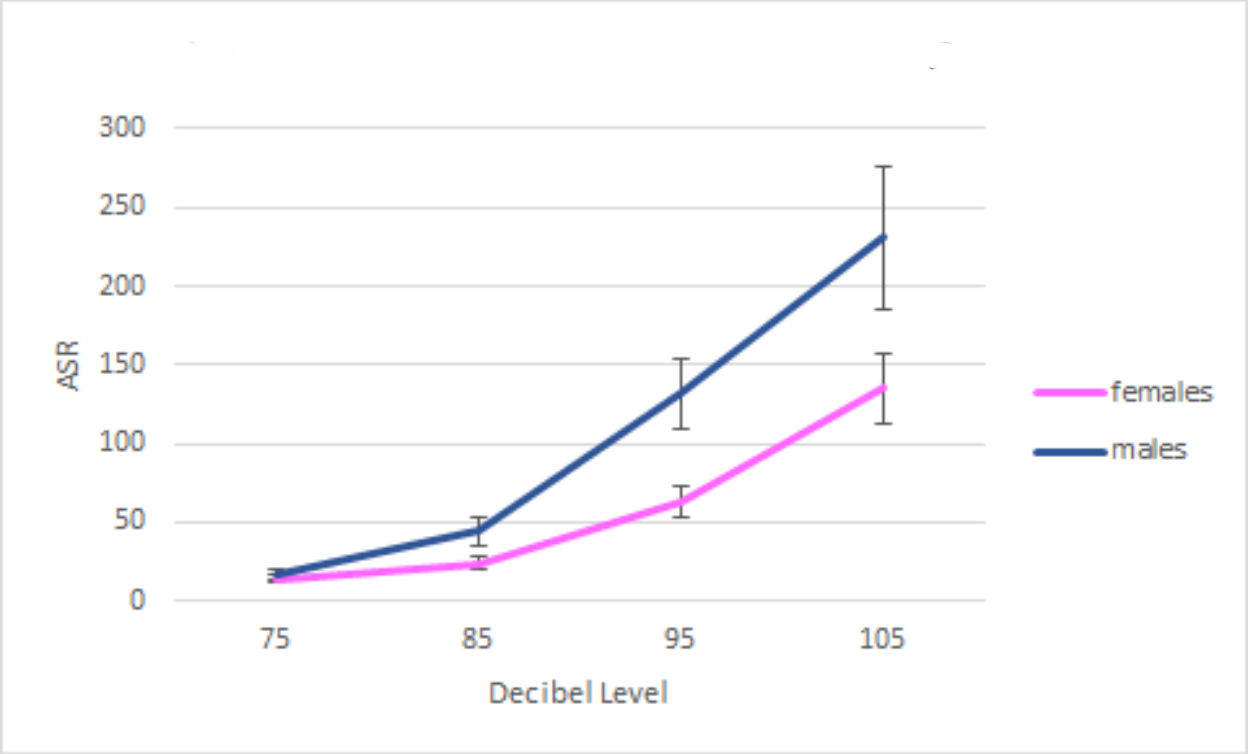

Figure 6: Average ASR by Sex Across Treatment Groups shows average ASR by decibel level and sex for PPA (n=7) and controls (n=8).

Acoustic Startle Response

ASR was tested as a measure of anxiety-like behavior in rats. PPA rats were hypothesized to have greater ASR in both the lights-off and lights-on conditions relative to controls as well as greater ASR in lights-on compared to lights-off. Before collecting data with PPA animals, untreated control animals (n=8) were run in the light-enhanced ASR test. We ran a two-way repeated measures ANOVA (see Figure 5) and found main effects of light (F_1=6.182, p<0.5) and decibel level (F_3=30.895, p<0.5) on ASR, as well as an interaction between light and decibel level (F_3=32.855, p<0.5). As was expected, ASR increased significantly with decibel level and lights on. These findings provide confidence in the setup of this experiment.

In evaluating the effects of PPA on ASR, 8 PPA animals and 7 controls were run. Unfortunately, a software malfunction prevented us from obtaining meaningful data from the lights-on portion of the study. Thus, the following results will pertain exclusively to the lights-off phase, or standard ASR. We ran a two-way repeated measure ANOVA and found that there was no significant difference of ASR between treatment groups F(3, 33)=40.780, p<0.5., refuting the hypothesis that PPA animals would exhibit greater ASR in lights-off. There was a sex difference F(1, 11)=4.801, p<0.5, with males startling more than females (See Figure 6). An interaction between decibel level and sex was also found; F(1, 11)=3.238, p<0.5, with significant differences in startle occurring at higher decibel levels.

Experiment Two

Crawley's Sociability Protocol

For each of the two stages, two independent samples T-tests were run. First, we compared distance from rats/empty cage in PPA animals and controls during SA stage. There was no significant difference between PPA animals (M=0.4, SD=0.1) and controls (M=0.5, SD=0.1) for empty cage; t(14)=0.411, p=0.659. There was no significant difference between PPA animals (0.3, SD=0.1) and controls (M=0.3, SD=0.1) for stranger rat; t(14)=-0.368, p=0.718. In the second independent samples t-test, we compared distance from rats/empty cage in females and males. There was no significant difference between females (M=0.4, SD=0.1) and males (M=0.5, SD=0.1) for empty cage; t(14)=-1.257, p=0.229 and no significant difference between females (M=0.4, SD=0.1) and males (M=0.3, SD=0.1) for stranger rat; t(14)=1.424, p=0.176.

For SN stage, we first compared distance from rats in PPA animals and controls. There was no significant difference between PPA animals (M=0.4, SD=0.1) and controls (M=0.4, SD=0.1) for stranger rat; t(14)=0.216, p=0.100. There was no significant difference between PPA animals (M=0.4, SD=0.2) and controls (M=0.3, SD=0.1) for novel stranger rat; t(14)=1.078, p=0.201. Next, we compared distance from rats in females and males. There was no significant difference between females (M=0.6, SD=0.1) and males (M=0.4, SD=0.1) for stranger rat; t(14)=-0.035, p=0.536. There was no significant difference between females (M=0.3, SD=0.1) and males (M=0.3, SD=0.1) for novel stranger rat; t(14)=-0.078, p=0.651.

Acoustic Startle and Light-enhanced Startle Response

ASR was assessed in IP animals. First, a two-way repeated measures ANOVA was run to assess effects of treatment and decibel on ASR (See Figure 8). There was an effect

of decibel on ASR; $F(3, 14)=56.441$, $p<0.05$ as startle increased with higher decibel levels. There was no effect of treatment; $F(1, 14)=0.089$, $p>0.05$. Another two-way repeated measures ANOVA was run to look at impact of sex and decibel level. As seen in Figure 9a, there was no effect of sex on startle; $F(1, 14)=0.908$, $p>0.5$. There was an effect of decibel; $F(3, 14)=52.646$, $p<0.5$. To verify that weight was not responsible for any potential startle differences, Pearson's correlation was determined for a distribution of weights and ASR for PPA animals (See Figure 9b). There was no correlation between startle and weight, $r=0.107$, $n=16$, $p=0.692$.

Data for LES was obtained for IP animals. Because 105 dB was found to produce the greatest startle response for ASR, average startle at 105 dB in both dark to dark and dark to light conditions were compared between treatment groups (See Figure 10). Light was found to have a main effect on startle; $F(1, 6)=38.116$, $p<0.05$, while there was no effect of the second dark session on startle; $F(1, 6)=0.136$, $p>0.05$. Thus, it can be concluded that startle increased due to light and not being in the startle chamber for a second session.

Additional Findings

During sociability testing, PPA animals displayed interesting periods of immobility. This observation was quantified using a one-way ANOVA which showed that PPA animals had a significantly greater average freezing score than controls; $F(1,14)=10.3954$, $p<0.05$ (See Figure 11).

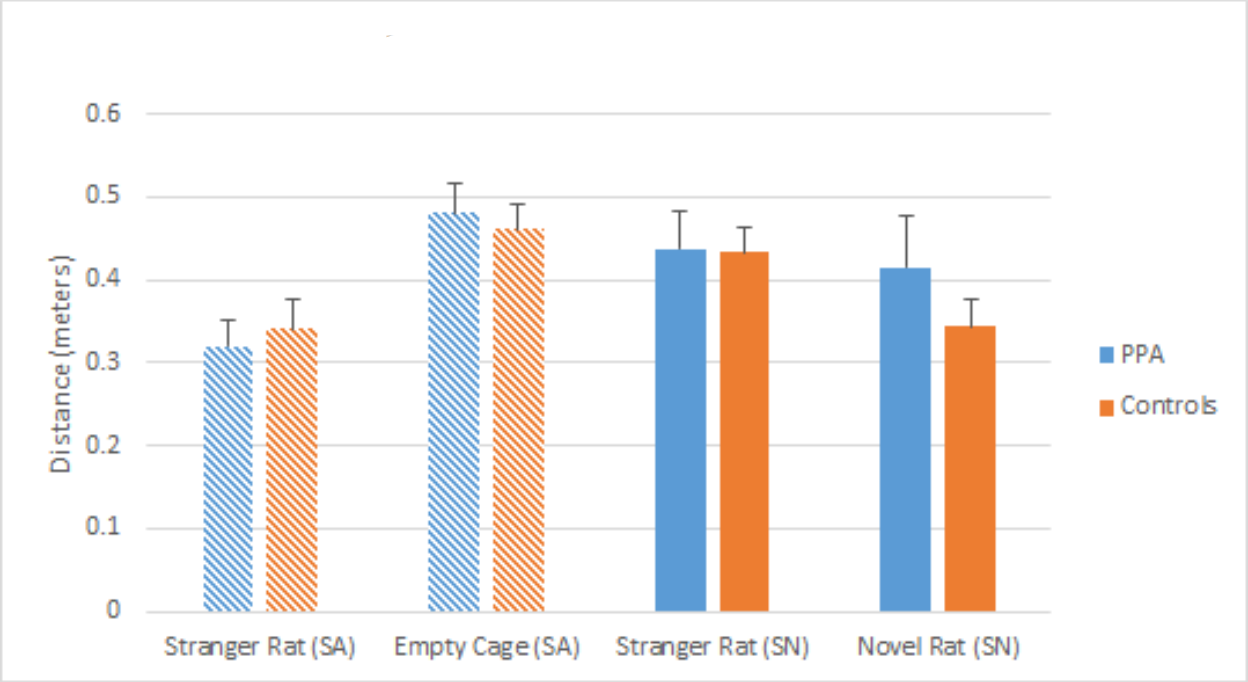

Figure 7: Average Distance from Rats shows average distance from stranger and empty cage/novel rat by stage or PPA (n=8) and controls (n=8).

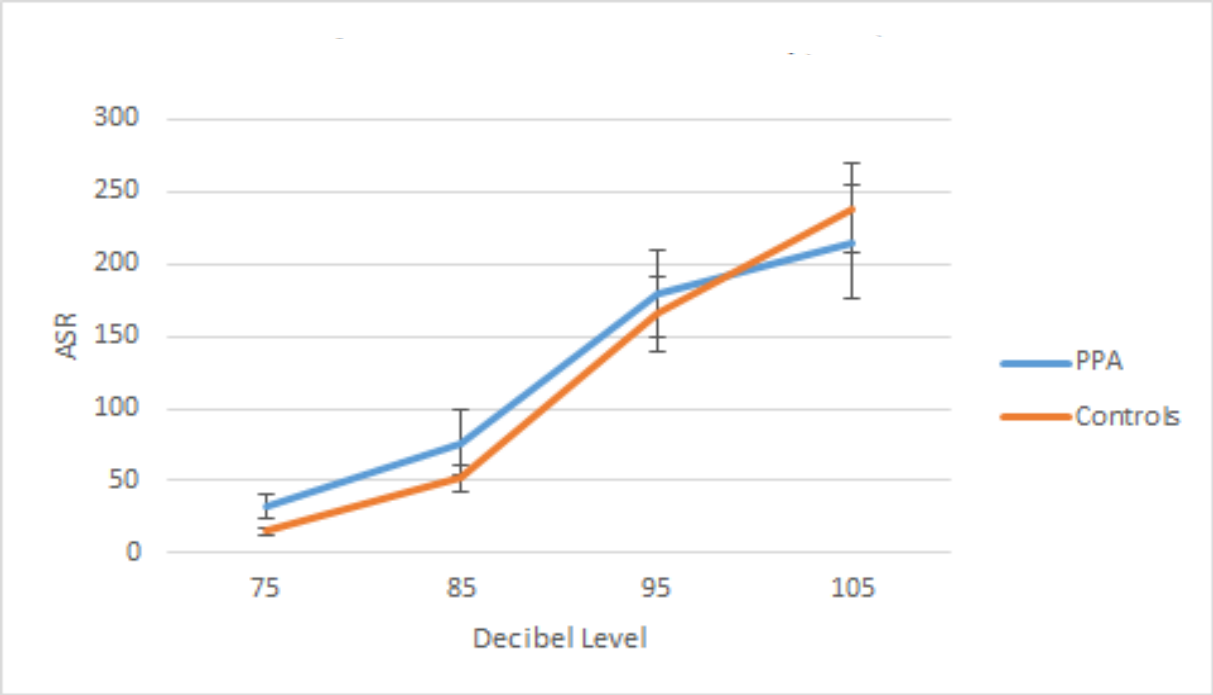

Figure 8: Average ASR by Decibel Level for PPA animals (n=8) and controls (n=8).

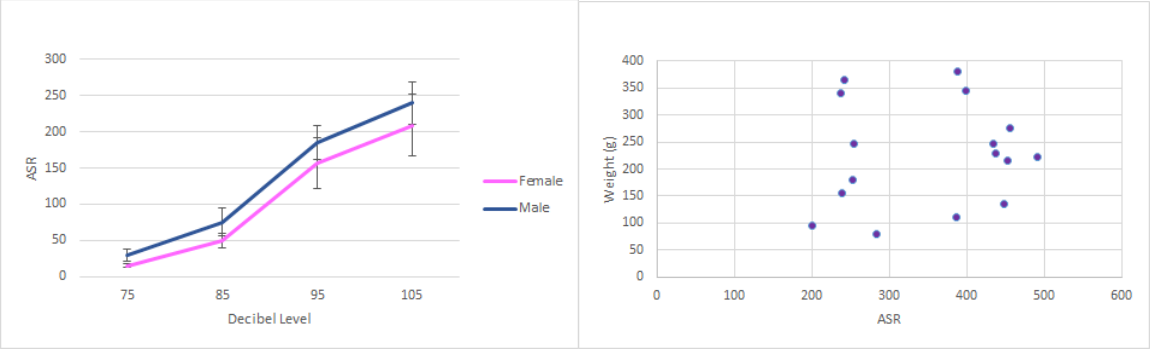

Figure 9a: Average ASR by Sex Across Treatment Groups shows average ASR by decibel level and sex for PPA animals (n=8) and controls (n=8). **Figure 9b: Distribution of ASR and Weight** shows a distribution of ASR and weight for PPA animals.

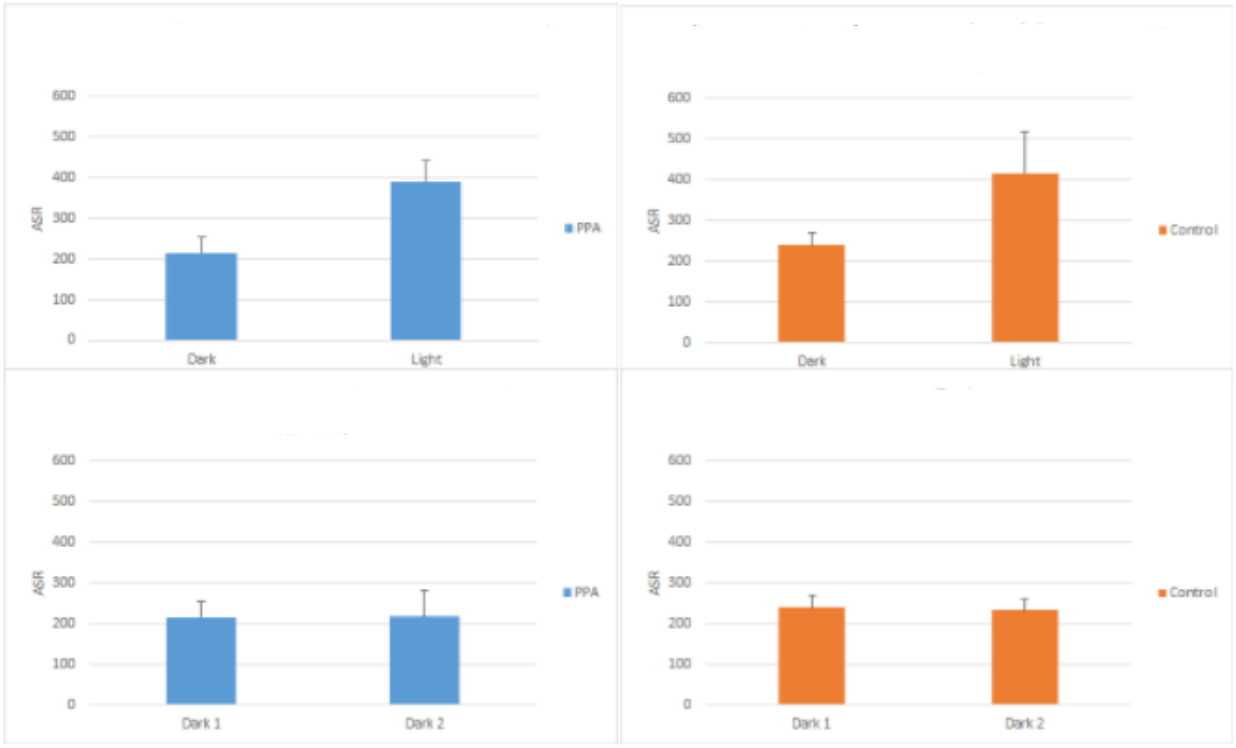

Figure 10: Average LES Response at 105 Decibels for PPA (n=8) and controls (n=8). The top left and right graphs show dark to light conditions for PPA and controls respectively. The bottom left and right graphs show dark to dark conditions for PPA and controls respectively.

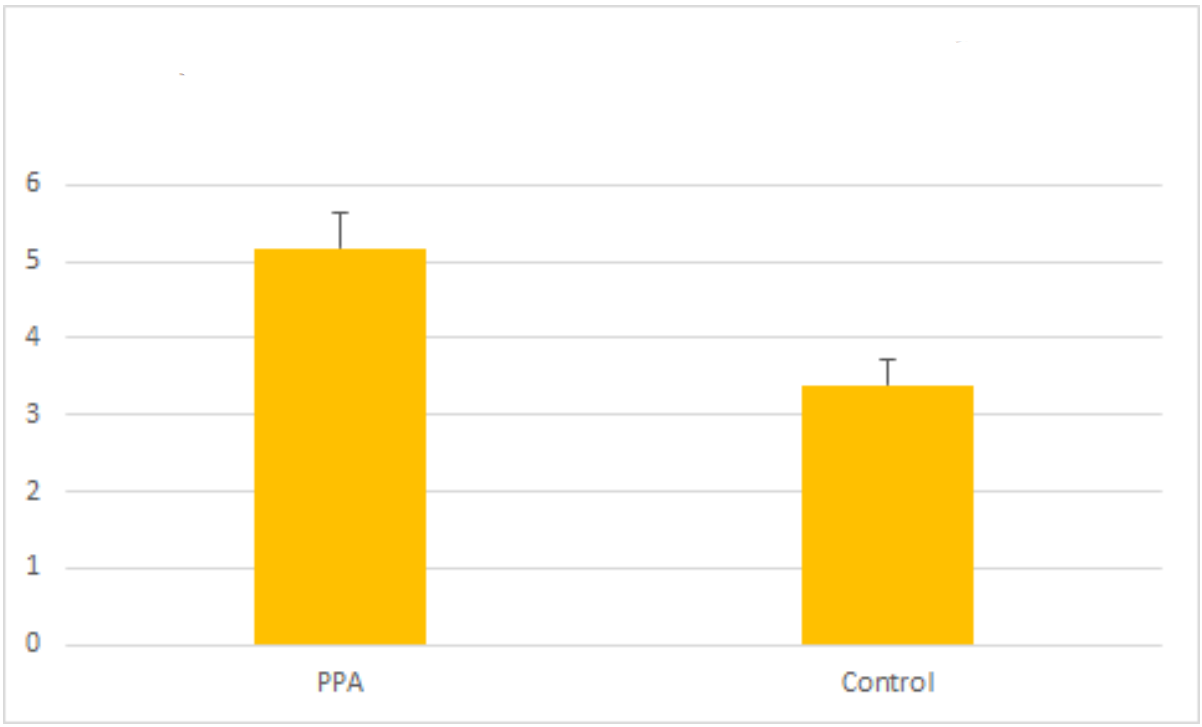

Figure 11: Average Immobility Scores During Sociability Protocol for PPA (n=8) and controls (n=8).

DISCUSSION

Autism is an increasingly prevalent disorder in society. To create meaningful treatments and solutions for symptoms, researchers seek to untangle the genetic, neurobiological, and environmental factors contributing to the disorders' complex and mysterious etiology. To do this, animal models of the disorder have been developed. One such model, the PPA rat model of autism has been shown to produce ASD-like behaviors and neurobiological changes seen in humans with the disorder. The PPA model is based on the idea that the gut-brain-axis contributes to the etiology of ASDs through elevated levels of gut microbiota that produce waste products capable of crossing the blood-brain barrier and triggering genetic and neurological changes resulting in ASD symptomatology. The gut-brain theory of ASDs is supported by studies observing high incidence of gastrointestinal issues in ASD populations, elevated PPA in stool, and the worsening of symptoms after eating foods high in PPA as a food preservative. It is also thought that synaptic dysfunction in both the CNS and the enteric nervous system contribute to the disorder. The purpose of this study was to further examine several aspects of the PPA rat model.

The results of Experiment One were unexpectedly insignificant. Hypothesized alterations in spatial cognition during MWM reversal training, decreased sociability and novelty preference, and increased ASR were not found. Anticipated impacts of sex on sociability and ASR were also lacking. This experiment employed ICV administration of PPA which has been shown to produce potent behavioral and biochemical ASD-like features in rats (Shultz et al., 2009). However, the present study used chronic

administration instead of the typically employed acute administration. Chronic administration of PPA over five days; as was done in this study, has been shown to create ASD-like seizure and motor dysfunction in rats (MacFabe, 2012). Moreover, five injections of PPA were found to produce less efficient swimming patterns in rats compared to conspecifics given three injections. Further, brain assays revealed markers of greater neuroinflammation in the PPA-5 group than the PPA-3 group. Thus, it was expected that chronic administration over five days would produce behavioral effects akin to those discovered with acute administration. While chronic administration has not been assessed for its impact on sociability or ASR, to the best of the author's knowledge, it was shown to impact MWM performance. Thus, it may be that chronic ICV administration does not impact these behaviors. It may also be that greater doses are required to create a potent behavioral effect. Future research should investigate the efficacy of a range of doses and duration of administration. There may be an effective combination of dose and duration that can produce behavioral effects when delivered chronically. A design weakness of this study was the lack of a positive control. Because we did not produce an acutely administered PPA model, we cannot say with complete certainty whether our drug worked or whether our design could produce behavioral impacts shown in previous studies. Another difference between the current study and previous research involving these behavioral tests, is the strain of rat used. This study made use of Sprague-Dawley rats, however, other studies used Long-Evans rats. Though these strains are extremely similar, the difference should be noted. It may also be that, had all the rats been male, differences in behavior would have reached statistical

significance. There are other possible explanations for the insignificant results of this study that will be discussed.

We chose to replicate the MWM test from Schultz et al. (2009) in an attempt to repeat the interesting perseveration findings from their acquisition and reversal training experiment. Unexpectedly, we did not find the same results. PPA animals did not demonstrate significantly longer search latencies than controls in either acquisition or reversal training.

This suggests several possibilities. First, these findings may be at least partially attributable to experimental error. Two rats had to be excluded from this test because they removed their skull caps and had to be euthanized, reducing the size of the control and experimental groups to six and seven respectively. It is also possible that some cannulas were not implanted properly, meaning that the drug was not delivered successfully to all experimental animals. However, these contradictory findings may ultimately mean that perseveration behavior is only produced by acute administration of PPA and not chronic administration. The rats from the Shultz et al. (2009) study did receive five injections over the course of experimentation, however, they were injected within a few minutes of testing. The rats of the present study were injected for five days straight before behavioral testing. In addition, MWM testing was done last, leaving several weeks in between administration and testing. This may suggest that the changes in latency and other aspects of spatial cognition pertinent to the MWM seen in the previous study by Shultz et al. (2009) are only generated by acute administration or dissipate after a certain time window. However, Shultz et al. (2009) also found that PPA-5 animals had greater

neuroinflammation than PPA-3 animals and were thus seemingly affected by a greater accumulation of PPA over time. From this, it stands to reason that repeated doses can create a long-lasting effect on behavior that the current study should have detected. While animals were given a week to recuperate after surgery, as recommended by previous literature to reduce the likelihood of seizure activity, the fact that there was no seizure activity at all may suggest that something went wrong in the development of the PPA model, inhibiting what should have been clear and potent behavioral impacts.

Our results yielded no significant difference between treatment groups for ASR. There are differences between the setup of the Foley et al. (2014) study and this study. Their study looked at adolescent rats with prenatal and both prenatal and postnatal injections of PPA. Thus, the rats treated postnatally had already been affected by prenatal injections. It is thus possible that, without the "double-hit" of prenatal and postnatal PPA, rats do not develop altered startle responses. However, it is also possible that PPA animals would show altered ASR in an acute administration set up, due to the 20-30 minute half-life of the drug (Foley et al., 2014). It is also possible that subcutaneous or other peripheral means of injection cause different or additional effects to those caused by ICV administration. Ideally, this experiment should be replicated with peripheral administration and at different time points over the rats' development. We did detect a significant sex difference as hypothesized. Males startled significantly more than females. Though this may be partially attributable to weight differences between males and females, it seems unlikely that weight is solely or predominantly responsible. As seen in Figure 9b, IP animals had no correlation between weight and startle. because ICV

animals were at a comparable age and likely weight to IP animals, it is likely that ASR findings are attributable to drug and not weight. Rather, ASR may present in a sexually dimorphic manner in the PPA model. However, the nature of our sex effect contradicts previous literature finding decreased startle in males and increased startle in females (Foley et al., 2014). This may reflect the variability of human ASDs and or the difference between administration routes between this study and previous work. The sex difference found in this study is further complicated by the results of ASR testing in Experiment Two which found no significant sex difference for ASR.

In addition to testing alternate administration schedules and types, we would like to repeat this experiment with the lights-on session working properly so that we can examine whether the added stimulus of bright light has additional impacts on ASR in PPA animals. Discerning what types of anxiety-like behaviors can and cannot be generated in animal models might help elucidate mechanisms of comorbid anxiety disorders in individuals with ASDs as well as the nature of sexually dimorphic symptom presentation in ASDs in general.

We hypothesized, based on previous literature and human ASD symptoms, that PPA animals would display decreased sociability and preference for novel strangers in a three-chamber sociability test. Our results did not support this hypothesis as, like the other behavioral tests, there was no significant differences between controls and PPA animals. Decreased sociability has been established in the model in previous literature, and so these results contradict expectations.

Because human females with ASDs typically display less social dysfunction than their male counterparts (Strasser, 2016), we hypothesized that males in the PPA condition would display greater decreases in sociability and novelty preference compared to females. This was not supported as there were no sex differences or apparent trends in our data in either the control or PPA groups to support this. This unexpected finding seems to contradict previous literature showing that there were marked social impairments in PPA males and that these were not displayed by PPA females (Benzaquen et al., 2010). It may be that the small sample size of males (n=4) and females (n=4) per treatment group was not statistically powerful enough to reach significance. It may also be that chronic administration of PPA does not engender sexually dimorphic sociability differences. This test should be replicated with greater numerical representation of both sexes in each treatment group and with a an acutely administered cohort for comparison.

Experiment Two assessed impact of PPA delivered intraperitoneally on sociability and ASR. It was expected that IP delivered PPA would result in decreased sociability and social novelty preference with males showing greater decrease than females. It was also hypothesized that PPA animals would have greater ASR and LES than controls and that males would startle more than females.

Sociability testing showed no significant difference between treatment groups for sociability or social novelty preference. This is consistent with ICV findings. Because PPA had no effect on sociability whether delivered directly to the brain or peripherally, these findings seem to indicate that chronic administration does not produce potent behavioral effects in the domain of social functioning. Interestingly, the IP animals

displayed greater periods of immobility during sociability testing, indicating that there was likely some kind of impact of the drug on behavior. Qualitatively, IP administered PPA animals were observed to have increasing aggression over the five days of injections conveyed by loud, unhappy vocalizations, writhing, and biting. This was most likely a product of the PPA and not merely an artifact of the injection procedure because controls receiving saline behaved typically. This may be further evidence that acute administration is necessary to create reliable behavioral effects because this increased aggression was not observed the week of testing. This suggests that any potential behavioral effects may have dissipated in the interim of administration and testing.

ASR testing revealed no significant difference between treatment groups for dark to dark (standard ASR) or light to dark (LES) conditions. This was consistent with ICV data. Unlike ICV results, however, IP animals showed no significant sex difference within the PPA group. This complicates any possible conclusions to be made from the impact of sex on startle. It may be that chronic PPA administration only creates sexually dimorphic startle responses in an ICV model and not through IP administration. However, it is difficult to conclude this without further replication of these experiments.

Studying the PPA model's ability to produce ASD-like symptoms and behaviors gives way to a larger discussion on the validity and usefulness of animal models in ASD research. Outside of ethical concerns, one might view animal models as unworthy investments because they do not completely capture human biology, nor does the research attached to them seem to translate into readily available solutions for individuals with immediate needs. While it must be acknowledged that an animal model cannot

perfectly embody a human disease or development, animal models of neurological disorders like ASDs can still be of use in understanding these conditions. Animal models allow researchers to study the underlying neurobiological mechanisms and etiologies of ASDs in ways that could never be done in humans with the ultimate goal of developing valid treatment options for humans struggling with severe deficits. As ASD research turns to fundamentally reversing the core symptoms of Autism that negatively impacts people's daily lives (Tania, Khan, & Xia, 2014), pharmacological treatments will need to be developed. To devise such treatments, valid animal models that can reliably present neurobiological and behavioral symptoms of ASDs and respond to treatments are necessary.

Animal model validity can be judged in three major components; face validity, construct validity, and predictive validity. An animal model with face validity is capable of producing behaviors relevant to and compromised in the same manner as in humans with the same condition. Construct validity refers to the model's ability to create relevant underlying neurobiological mechanisms of the condition. A model has predictive validity if successful condition interventions similarly ameliorate symptoms in the model. Because of the incredible variability in ASDs and the fact that the ultimate etiology is still unknown, is it difficult to say whether one has truly established a model with face validity. In the case of the PPA model, some researchers wonder if the model is realistic enough given that relatively high doses of the drug are needed to create potent effects. And, limited studies elevating endogenous PPA in rats by administering an antibiotic that promotes PPA-producing microbiota growth, rather than injecting PPA directly into the

brain, does not seem to produce significant behavioral symptoms (Al-Ghamdi, Al-Ayadhi, & El-Ansary, 2014).

Despite this area of questioning and ongoing investigation, the PPA model has been shown to reliably produce ASD-like behavioral impairments and is thus considered to have robust face validity. Its construct validity is also considered sound, given that the drug seems to affect neurobiological elements similarly affected in humans with ASDs. Of note are its ability to create neurological inflammation and oxidative stress as well as altering expression of dopamine, serotonin, GABA, and glutamate systems similarly to that observed in human ASDs (Al-Ghamdi, Al-Ayadhi, & El-Ansary, 2014). In terms of predictive validity, there have been promising studies finding possible therapeutic agents for ASD-like features in the model. Al-Ghamdi, Al-Ayadhi, and El-Ansary (2014) found that CoQ and melatonin had protective, therapeutic impacts on PPA rats, significantly increasing neurotransmitter levels that PPA had decreased. Al-Salem, Bhat, Al-Ayadhi, and El-Ansary (2016) found that bee pollen could similarly ameliorate biochemical ASD features including oxidative stress, neuroinflammation, and abnormal gut microbiota composition in rats given oral PPA.

Animal models allow for researchers to work with and understand disorders and diseases in the larger context of a complex living system. Purely studying neurons in a petri dish would not inform on the behavioral effects related to alterations in these neurons and leave researchers with an incomplete picture of the disorder of interest. This is an especially important consideration when investigating neurodevelopmental disorders with overt behavioral profiles. Animal models at large have been historically

essential to developing vaccines and medicinal treatments for many diseases and disorders. Humanized mouse models, for example, have been used successfully in human cancer research, especially in grafting tumors (Denayer, Stohr, & Van Roy, 2014).

In conclusion, the PPA model of ASDs is an overall relatively robust and useful animal model for studying the disorders. Further research should continue discerning the efficacy of various routes of administration in creating relevant behavioral profiles. Our unexpected lack of significant findings in spatial cognition, sociability impairment, and startle response seemingly suggest that chronic administration of PPA may not produce significant behavioral symptoms in rats. This suggests that the model may not be useful for looking at long term behavioral impairment and may pose difficulties in developing chronically administered treatments which more closely resemble human treatment. However, previous literature shows that five cumulative doses of PPA create more significant neurobiological and behavioral impairments in rats than three doses (MacFabe, 2012). Thus, there may still be a cumulative effect of multiple PPA injections that the current study did not detect. It may also be that acute administration may be required to bring out behavioral effects of the drug. More research into the differences in chronic and acute administration of PPA should be performed to better understand these disparities. Continuing work with the PPA model will hopefully help researchers better understand the mechanisms of the gut-brain axis and its relations to the development and symptoms of ASDs in humans.

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