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ORIGINAL ARTICLE

Impaired Spatial Cognition in Adult Rats Treated with Multiple Intracerebroventricular (ICV) Infusions of the Enteric Bacterial Metabolite, Propionic Acid, and Return to Baseline After 1 Week of No Treatment: Contribution to a Rodent Model of ASD

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Abstract

Propionic acid (PPA) is a dietary short chain fatty acid and an enteric bacterial metabolite. Intracerebroventricular (ICV) infusions of PPA in rodents have been shown to produce behavioral changes similar to those seen in autism spectrum disorders (ASD), including perseveration. The effects of ICV infusions of PPA on spatial cognition were examined by giving rats infusions of either PPA (0.26 M, pH 7.4, 4 μl/infusion) or phosphate-buffered saline (PBS, 0.1 M) twice a day for 7 days. The rats were then tested in the Morris water maze (MWM) for acquisition of spatial learning. After a recovery period of 1 week of no treatment, the rats were then tested for reversal of spatial learning in the MWM. PPA-treated rats showed impaired spatial learning in the maze, relative to controls, as demonstrated by increased search latencies, fewer direct and circle swims, and more time spent in the periphery of the maze than PBS controls. After a recovery period of 1 week of no treatment, these animals exhibited normal spatial reversal learning indicating that the behavioral cognitive deficits caused by PPA seem to be reversible.

Keywords Autism spectrum disorders · Animal model · Spatial cognition · Perseveration · Short chain fatty acid · Rat

Introduction

Autism spectrum disorders (ASD) are a family of lifelong disorders characterized by communication deficits, social impairments, and restricted and/or ritualistic behaviors (Arndt et al. 2005; DiCicco-Bloom et al. 2006). A subset of patients also present with co-morbidities, such as gastrointestinal disturbances and seizure disorder (Besag 2004; Horvath et al. 1999). Additionally, human autopsy studies of patients with autism have reported central nervous system (CNS) immune activation in the form of increases in reactive astrogliosis and activated microglia (Vargas et al. 2005).

Research has shown that there is a strong genetic contribution to ASD (Bailey et al. 1995), but recent evidence suggests that environmental factors may play a key role (Benach et al. 2012; De Angelis et al. 2013; Dietert et al. 2011). Lack of complete concordance rates among monozygotic twins, along with notable variation in severity of the disorder, even when both twins are affected, suggests that genetics are not solely responsible for the disorder (Hu et al. 2006). Many environmental factors have been implicated in this disorder, which is now widely considered to be a condition involving immune, digestive, and metabolic dysfunction; all of which may be triggered by environmental factors in genetically susceptible individuals (Ashwood and Van de Water 2004; Chauhan and Chauhan 2006; Herbert et al. 2006; Horvath and Perman 2002; Frye et al. 2015). Several of these putative environmental contributors have been studied, including pre- and postnatal exposure to valproic acid (Ingram et al. 2000), ethanol (Arndt et al. 2005), and thalidomide (Narita et al. 2002). Similarly, exposure to certain metals and viral infections has

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been implicated (Curtis et al. 2010; Fatemi et al. 2000; Patterson 2009). Beyond these studies, support for an environmental hypothesis also comes in the form of anecdotal reports of the worsening of autistic symptoms following gastrointestinal abnormalities (Horvath et al. 1999) and/or treatment with antibiotics (Finegold et al. 2002; Fallon 2005). Similarly, ingestion of certain wheat or dairy products has also been shown to exacerbate symptoms. A subset of autistic patients has shown improvements in the occurrence and severity of symptoms once these dietary components have been removed from their diet (Jyonouchi et al. 2002).

Based on the whole-body nature of ASD (Herbert et al. 2006), as well as the putative involvement of a variety of different environmental factors, recent research has focused on animals models that tackle specific characteristics of ASD. The administration of propionic acid (PPA), as well as other short-chain fatty acids (SCFA), has been proposed as a novel model for ASD in the rat (MacFabe et al. 2007, 2008, 2011; Ossenkopp et al. 2012; Shultz et al. 2008, 2009; Thomas et al. 2010). PPA is a SCFA that is an important metabolic fermentation product of some enteric gut bacteria (Al-Lahham et al. 2010; Finegold et al. 2002, 2010). Being a weak organic acid, PPA exists in both water-soluble and lipophilic forms in the body and can readily cross lipid bilayers (such as the gut-blood and blood-brain barriers), gaining entry into systemic and/or CNS environments. This can occur either actively, via monocarboxylate transporters, or passively, through diffusion (Niederman et al. 1997; Maurer et al. 2004). Although PPA is necessary for normal immune and physiological functioning, elevated levels may result in disruptive effects (Al-Lahham et al. 2010; Brestoff and Artis 2013).

PPA has been investigated in a potential adult rodent model of ASD. Central (ICV) administration of PPA has been shown to impair social behavior and some cognitive tasks, induce convulsions and seizures, as well as induce an innate neuroinflammatory response and oxidative stress in the brains of treated adult rats (MacFabe 2012; MacFabe et al. 2007, 2008, 2011; Shultz et al. 2008, 2009; Ossenkopp et al. 2012). This adult model is based on the premise that continuous high levels of PPA could be responsible for some of the phenotypic behavioral abnormalities seen in ASD. This premise is supported by previous studies showing that propionic acidemia and ASD overlap in a number of patients. Propionic acidemia, a neurodevelopmental metabolic disorder characterized by elevated levels of the SCFA PPA, clinically resembles some aspects of autism (Feliz et al. 2003), and case studies of comorbidity of propionic acidemia and ASD have been presented (Al-Owain et al. 2012; de la Batie et al. 2018; Witters et al. 2016). In addition, behavioral effects of systemic treatment with PPA, such as changes in acoustic startle response levels (Kamen et al. 2019), have been shown to be dose dependent, with greater suppression of startle response magnitude occurring with higher doses of PPA treatment.

PPA is known to have many physiological effects, acting on processes, such as cell signaling (Nakao et al. 1998), neurotransmitter synthesis and release (DeCastro et al. 2005), immune function (Le Poul et al. 2003; Wajner et al. 1999), modulation of gene expression (Suzuki et al. 1996), mitochondrial function (Wagner et al. 2004), lipid metabolism (Hara et al. 1999), and gating of gap junctions (Rorig et al. 1996); all of which have been implicated in ASD (Koh et al. 2016; MacFabe 2012, 2015). Beyond this wide array of physiological effects of PPA, further evidence for the role of SCFAs in the pathogenesis of autism comes from the increased prevalence of PPA-producing bacterial species in the intestinal tracts of autistic patients compared to healthy controls (Kang et al. 2018; Finegold et al. 2002; Wang et al. 2012). Finally, altered PPA metabolism is present in many disorders, such as organic acidemias, carnitine/B12/biotin deficiency, and exposure to valproate or ethanol (pre- or postnatally); all of which present with ASD-like characteristics, such as developmental delay, GI difficulties, and seizure disorder (Coulter 1991; Calabrese and Rizza 1999; Feliz et al. 2003; Wagner et al. 2004).

Findings using the PPA rodent model of autism have included behavioral, neuropathological, biochemical, and electrophysiological characteristics; all of which are consistent with those seen in ASD (see MacFabe 2012, 2015). Behaviorally, traits, such as bouts of hyperactivity, social impairments, perseveration, reduced auditory startle reaction, and object preference (Kamen et al. 2019; MacFabe et al. 2007, 2008, 2011; Shultz et al. 2008; Wah et al. 2019), have been observed. Electrophysiological findings have shown both caudate spiking and limbic kindled seizures (MacFabe et al. 2007). Upon examination of neuropathological data obtained using this model, a clear innate neuroinflammatory response as well as an increase in oxidative stress markers and a reduction in glutathione were seen in the brains of these rats (MacFabe et al. 2007, 2008), which have also been noted in autopsy data from human patients with ASD (Chauhan and Chauhan 2006; Vargas et al. 2005). Developmental studies on the pre- or post-natal effects of PPA in rats have also found behavioral and neuropathological abnormalities consistent with ASD (Choi et al. 2018; El-Ansary et al. 2013; Foley et al. 2014a, b, 2015; Shams et al. 2018).

In 2009, Shultz and colleagues investigated the effects of brief ICV infusions of PPA on the cognitive and sensorimotor functioning of Long-Evans rats, using the Morris water maze (MWM). Animals were given either three or five ICV infusions and then tested in the MWM for acquisition, and then again a week later in a maze reversal task. What was found was a highly unusual pattern of water maze performance, wherein rats treated with PPA were able to learn the maze during acquisition just as well as controls, but were unable to learn the maze reversal task (i.e., learn a new location of the hidden platform) due to perseveration in returning to the original quadrant where the platform was initially located

(Shultz et al. 2009). This perseverative pattern of activity is consistent with the ritualistic and repetitive behaviors seen in autism (Sasson et al. 2008). This finding was corroborated by the fact that MacFabe et al. (2007) found increased immunoreactivity of the activated form of cyclic AMP responsive binding protein (CREB) in the tissue of the hippocampus and adjacent white matter. CREB and pCREB (the phosphorylated and activated form) are involved in the alterations of gene expression that are thought to be important in learning and memory (Carlezon et al. 2005; Silva et al. 1998). The current study further investigated the influence of ICV infusions of PPA on MWM learning in this rodent model.

A 7-day infusion schedule, with two infusions per day, was used in the current study, in order to be able to compare the findings to some of the previous work done using the PPA rodent model of autism (MacFabe et al. 2007, 2008; Thomas et al. 2010). The present study thus differed from the previous study by Shultz et al. (2009), where animals were given either three or five infusions over the course of the experiment. In the present study, animals were also allowed a 7-day period of recovery (no infusions were administered) following the initial 7-day infusion schedule. It was hypothesized that while certain aspects of behavior and cognition might persist following PPA treatment, many of the behavioral effects caused by PPA administration would return to baseline after a period of recovery because the systemic levels of PPA would have dropped over the recovery period.

Methods

Subjects

Subjects were 41 adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada) and weighed between 200 and 250 g at the start of the experiment. Rats were housed in pairs in standard acrylic cages (26 cm × 48 cm × 21 cm) in a temperature controlled colony room (21 ± 1.0 °C) and were naïve to all experimental procedures. After surgery, rats were housed individually and allowed to recover for 7–14 days, with no treatment taking place during this time. The light/dark schedule was a 12:12 cycle with lights on at 07:00 h, and animals were allowed access to food (ProLab RHM3000 rat chow) and tap water ad libitum. All test procedures and experimentation were carried out in the light phase and were in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and approved by the Western University Animal Use Committee.

Surgery: Cannula Implantation

Animals were implanted with a 23-gauge intraventricular guide cannula using standard stereotaxic techniques. Rats were anesthetized using inhaled 5% isoflurane and 2-L/min oxygen flow. While in the stereotaxic device, rats were equipped with a gas anesthesia nose cover to maintain anesthetic throughout surgery with 3% isoflurane and 500-mL/min oxygen flow. Under aseptic conditions, rats were implanted with a guide cannula in the right lateral ventricle, with the tip of the guide cannula at the following coordinates with reference to Bregma: anterior/posterior − 1.4 mm; medial/lateral 1.8 mm; dorsal/ventral − 3.0 mm (Paxinos and Watson 1986). Four small, stainless steel screws were inserted into the skull surrounding the cannula to provide anchors for dental acrylic, which attached the cannula to the skull. The cannula was sealed with a removable plug until the time of infusion. Immediately post-surgery, all rats received a subcutaneous injection of ketoprofin (1 mL/kg) as an analgesic.

Treatment Groups

In phase I, rats were randomly assigned to two groups; one group received ICV infusions of phosphate-buffered saline (PBS; 4 μL of 0.1-M solution, $n = 16$) or PPA (4 μL of 0.26-M solution, $n = 25$). PPA was buffered to a physiological pH of 7.4 using hydrochloric acid or sodium hydroxide. During phase I, animals received two infusions of their assigned substance per day, at 09:00 and 13:00 h, for 7 days. Following these 14 infusions, the rats were allowed a period of rest for 7 days, during which no treatments were given. In phase II, these two groups were randomly subdivided to yield four treatment groups. The rats receiving PBS during phase I followed by PBS during phase II were labeled PBS-PBS ($n = 7$). Rats receiving PPA during phase I and PPA during phase II were labeled PPA-PPA ($n = 12$). Rats in the PPA-PBS group received PPA during phase I and PBS during phase II ($n = 13$) and vice versa for rats in the PBS-PPA group ($n = 9$).

On the day following the rest period (phase II), rats were given two more infusions of either the same compound that they received during phase I (PBS-PBS and PPA-PPA groups), or of the compound they did not receive during phase I (PPA-PBS and PBS-PPA groups). The dose of PPA used in the present experiment was based on previous dose-response findings from earlier studies done in our laboratory (MacFabe et al. 2007; Shultz et al. 2008).

ICV Infusions

Each rat received infusions of its assigned compound directly into the right lateral ventricle via a 30-gauge injection cannula connected to a Sage syringe pump by PE10 tubing. The tip of the injection cannula protruded 0.5 mm beyond the tip of the guide cannula. Each infusion consisted of 4 μL of solution delivered over a period of 1 min. To ensure that the entire infusion had been delivered, the injection cannula was allowed to remain in place for an additional minute before

being removed. On behavioral testing days, infusions were given approximately 3–5 min prior to the test session.

Behavioral Test Apparatus

Open Field Apparatus

Locomotor activity was monitored in a circular open-field arena (90-cm diameter, 40-cm high) with Beta Chip bedding covering the floor of the arena (Ossenkopp and Kavaliers 1996). A CD camera and a darkroom lamp were mounted above the center of the arena. The camera was connected to a computer, allowing behavior to be recorded using the *EthoVision 3.0.15 Behavioral Monitoring and Analysis System* at a rate of 5.994 frames/s. This program is capable of tracking the *x-y* coordinates of each animal, and it allows for the computation of several quantitative variables. The camera was also connected to a VCR, allowing behavior to be recorded for later analysis.

Morris water maze

Spatial cognition was assessed using a water maze consisting of a circular pool (1.5 m in diameter, 45-cm deep) filled with tap water at 29 ± 1.0 °C. Hidden approximately 2 cm below the water surface was an escape platform (9 cm × 9 cm). The platform was located in the center of the south-east quadrant during acquisition (phase I) and in the center of the north-west quadrant during reversal (phase II). Polypropylene pellets floating on top of the water prevented the rats from seeing the hidden platform by making the surface opaque (Cain et al. 1993). Pictures and objects around the room provided a variety of distal cues. Behavior was recorded by a video camera mounted to the ceiling above the pool. The camera was connected to a computer and behavior was objectively analyzed by an automated tracking system that digitized each swim trial (*Poly-Track, San Diego Instruments*, San Diego, CA).

Experimental Procedure

Phase I

During phase I, rats received infusions of their assigned drug twice a day for 7 days. On the final day of infusions, day 7, the rats were tested in the open field apparatus in the morning, following their first infusion. Each rat was placed individually into the open field, and behavioral data were collected for 30 min. In the afternoon, following the second infusion of the day, rats were tested in the water maze. Behavioral testing commenced within approximately 3–5 min of the infusion.

Rats were given 10 training trials in the water maze. Each trial began with the rat being placed in the pool, adjacent to, and facing the pool wall at one of four quasi-random start locations (north, east, south, or west) and ended when the rat stood on the hidden platform. The platform was located in the center of the south-east quadrant during maze acquisition. Rats that failed to reach the hidden platform within 60 s of the commencement of the trial were guided to the platform by the experimenter. Rats remained on the platform for 15 s before they were placed in a drying chamber that was heated from above by an infrared lamp. Due to metabolic clearance rates of the injected substances (approximately 30 min; Brusque et al. 1999) rats were run in squads of 4 so that the inter-trial interval for the 10 acquisition trials was not more than 5 min. For graphic presentation of water maze variables, the time to reach the platform was averaged for every block of two trials (e.g., block 1 = (trial 1 + trial 2) / 2). Following acquisition training in the water maze, rats were given no treatment for 1 week.

Phase II: Reversal Training

Phase II consisted of 1 day of infusions (at 09:00 and 13:00 h) and testing and occurred 1 week after the conclusion of phase I. Rats again received two infusions of either PBS or PPA, with half of the rats receiving the infusion type (PBS or PPA) that they had not received during Phase I (i.e., PPA-PBS and PBS-PPA groups). Following the morning infusion, rats were once again placed in the open field apparatus for 30 min, and behavioral data were collected. After the afternoon infusion, the rats were again tested in the water maze, following the same procedure as on the seventh day of phase I. However, the platform was now located in the north-west quadrant of the pool rather than the south-east quadrant, where it was located during maze acquisition.

The day after the reversal test day (phase II), rats were deeply anesthetized with sodium pentobarbital (270 mg/mL) and perfused transcardially with ice cold 0.1-M phosphate-buffered saline (PBS, pH 7.5) followed by 4% paraformaldehyde in PBS. Coronal brain sections along the cannula track were cut using a cryostat, and then mounted on glass slides. Microscopic examination of the cresyl violet stained sections confirmed that all cannula placements were in the right lateral ventricle.

Statistical Analyses of Behaviors

All statistical tests were calculated using SPSS 18.0 (SPSS, Inc.) for Windows. Tests were completed using $\alpha = .05$ as the criterion for significant effects.

Water Maze Search Latencies, Total Distance Traveled, Time Spent in Periphery, Swim Speed, Number of Direct and Circle Swims, and Percent First Choices to Initial Quadrant

Search latency was defined as the time in seconds from release until the rat climbed onto the hidden platform. Time spent in the periphery was calculated with the periphery being defined as the outer 33% of the pool. A direct swim was defined as a swim that remained entirely within an 18-cm wide virtual alley from the start point to the hidden platform without crossing over itself. A circle swim was defined as a swim that approximated an arc of a circle without exceeding 360° or crossing over itself. Direct and circle swims are indicative of spatial place learning and were summed for each test session.

Data were analyzed using a mixed design analysis of variance (ANOVA) with drug treatment (PBS or PPA) as the between-subjects factor and trial (10 training trials) and test day (one or two) as the within-subjects factor. Student-Newman-Keuls (SNK) post hoc tests were carried out to obtain group differences on individual trials.

Open Field Total Distance Traveled, Average Speed, and Time Spent in Periphery

Data were analyzed for main effects using a mixed design ANOVA with drug treatment (PBS or PPA) as the between-subjects factor and time bin (six 5-min time bins) and test day (one or two) as the within-subjects factor.

Monitoring of Convulsive Activity

Rats were closely monitored for possible convulsive behavior. Past studies from our lab have found a kindling effect associated with repeated daily ICV infusions of PPA in some rats (MacFabe et al. 2007). The onset of seizures may be an issue in studies investigating cognition or sensorimotor ability if a seizure was to occur prior to, or during testing.

Results

Baseline Locomotor Activity

There were no significant group differences among the drug groups (dummy variables) for any of the behavioral variables measured prior to drug treatment.

Open Field

Total Distance Traveled In the open field apparatus, all rats traveled significantly less across time (habituation), $F(5, 195) = 25.405$, $p < .001$. The PPA-treated group exhibited significantly greater distances traveled than PBS controls, $F(1, 39) = 19.716$, $p < .001$, as seen in Fig. 1a. There was no significant interaction between time and drug, $F(5, 195) = 2.034$, $p = .328$.

On reversal day, all rats once again traveled significantly less across time in the open field, $F(5, 185) = 13.863$, $p < .001$. There was no significant main effect of drug, however, $F(3, 37) = 1.283$, $p = .295$ (Fig. 1b).

Average Speed

All rats tended to travel at a higher speed in the beginning of the session compared to the end, $F(5, 195) = 103.720$, $p < .001$ (Fig. 2a). The PPA-treated group exhibited significantly greater average speeds than PBS controls across all time bins, $F(1, 39) = 19.433$, $p < .001$. There was no significant interaction between time and drug, $F(5, 195) = 1.666$, $p = .171$.

On reversal day, all rats once again traveled at significantly lower average speeds across time in the open field, $F(5, 185) = 83.483$, $p < .001$ (Fig. 2b). There was no significant main effect of drug, however, with all groups traveling at similar speeds, regardless of drug treatment, $F(3, 37) = .862$, $p = .469$.

Time Spent in Periphery

In the open field apparatus, all rats spent less time in the periphery of the arena across time, $F(5, 185) = 11.891$, $p < .001$. However, there was no significant main effect of drug, $F(1, 37) = 2.668$, $p = .111$. There was also no significant interaction between time and drug, $F(5, 185) = 1.161$, $p = .328$ (Fig. 3a).

On reversal day, all rats once again spent significantly less time in the periphery of the open field over the course of the testing session, $F(5, 185) = 19.756$, $p < .001$ (Fig. 3b). There was no significant main effect of drug, $F(3, 37) = .352$, $p = .788$.

Water Maze

Removal of Data from Convulsive Rats

Throughout the experiment, several rats experienced convulsive activity during acquisition of the water maze, which occurred immediately following infusion. This is a common feature of rats subjected to this particular infusion schedule. Because of the nature of the task, the data collected from these rats was excluded from water maze analyses. In total, water maze data from 16 rats were excluded.

Search Latencies

During acquisition training, all treatment groups exhibited decreased search times as training progressed. Significant main

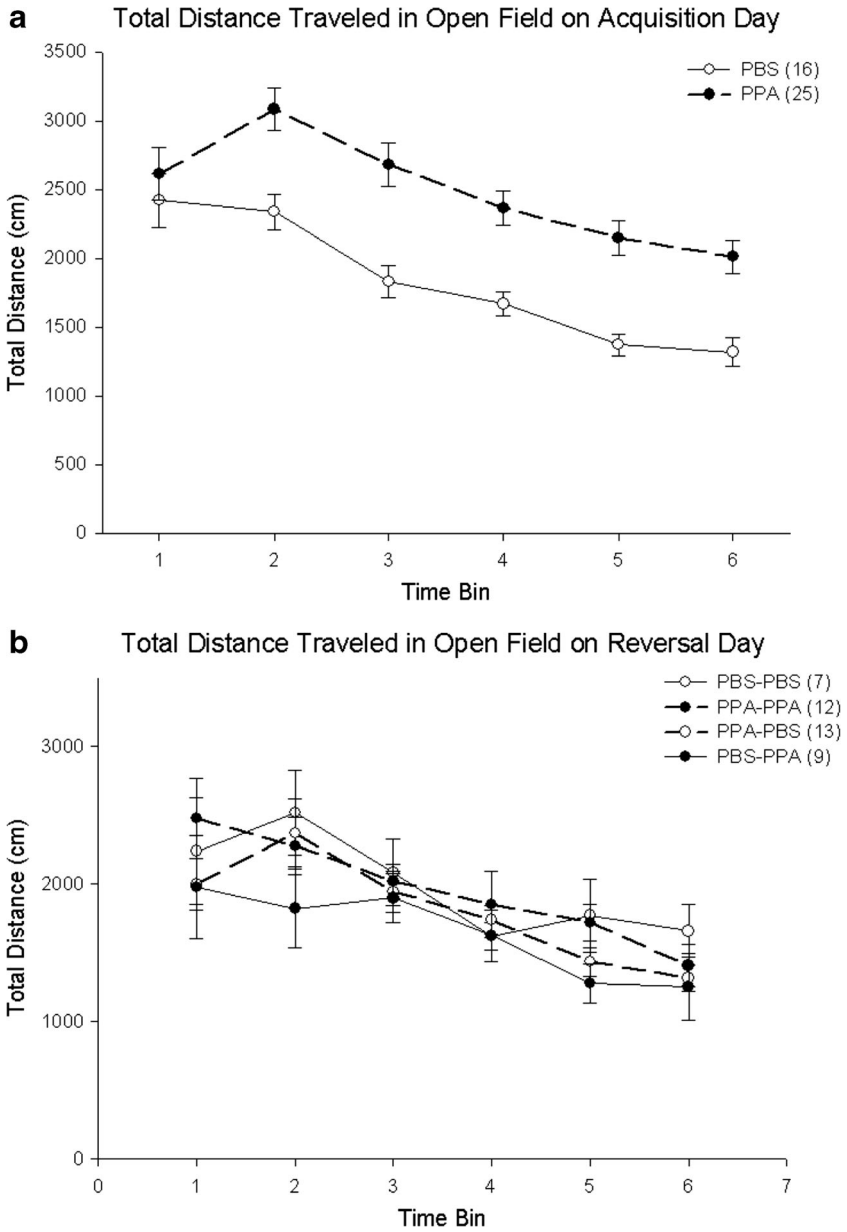

Fig. 1 Total distance (cm) traveled in open field on Acquisition day (**a**) and total distance traveled on reversal day (**b**) for rats injected (ICV) with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each time bin in the 30-min session immediately following injection. Error bars represent ±SEM. PPA-treated animals traveled significantly greater distance on acquisition day, but there were no significant group differences found on reversal day

effects were found for both trial, $F(9, 207) = 11.421$, $p < .001$, and drug treatment $F(1, 23) = 14.755$, $p < .01$. There was also found to be a significant interaction between trial and treatment, $F(9, 207) = 3.536$, $p < .01$, indicating that PPA-treated rats exhibited longer search latencies on blocks 2, 3, 4, and 5 (Fig. 4a).

On reversal day, as shown in Fig. 4b, all treatment groups exhibited decreased search times as training progressed, $F(9, 189) = 8.084$, $p < .001$. However, there were no significant differences found among groups on reversal day $F(3, 21) = 2.421$, $p = .061$. There was also found to be no significant interaction between trial and treatment, $F(27, 189) = .703$, $p = .686$, indicating that the improvement across trials was similar for each drug group.

Total Distance Traveled

During acquisition training, all treatment groups exhibited decreased distances traveled as training progressed, as seen in Fig. 5a. However, distances traveled by PPA-treated rats decreased less than the PBS controls. These impressions were confirmed by ANOVA, with significant main effects being found for both trial, $F(9, 189) = 10.143$, $p < .001$, and drug treatment $F(1, 21) = 19.212$, $p < .001$. There was also found to be a significant interaction between trial and drug treatment, $F(9, 189) = 3.971$, $p < .01$, indicating that the decrease in total distance traveled across trials was greater in the PBS group than in the PPA group. PPA-treated rats continued to travel larger distances compared to controls on trial blocks 2 through 5.

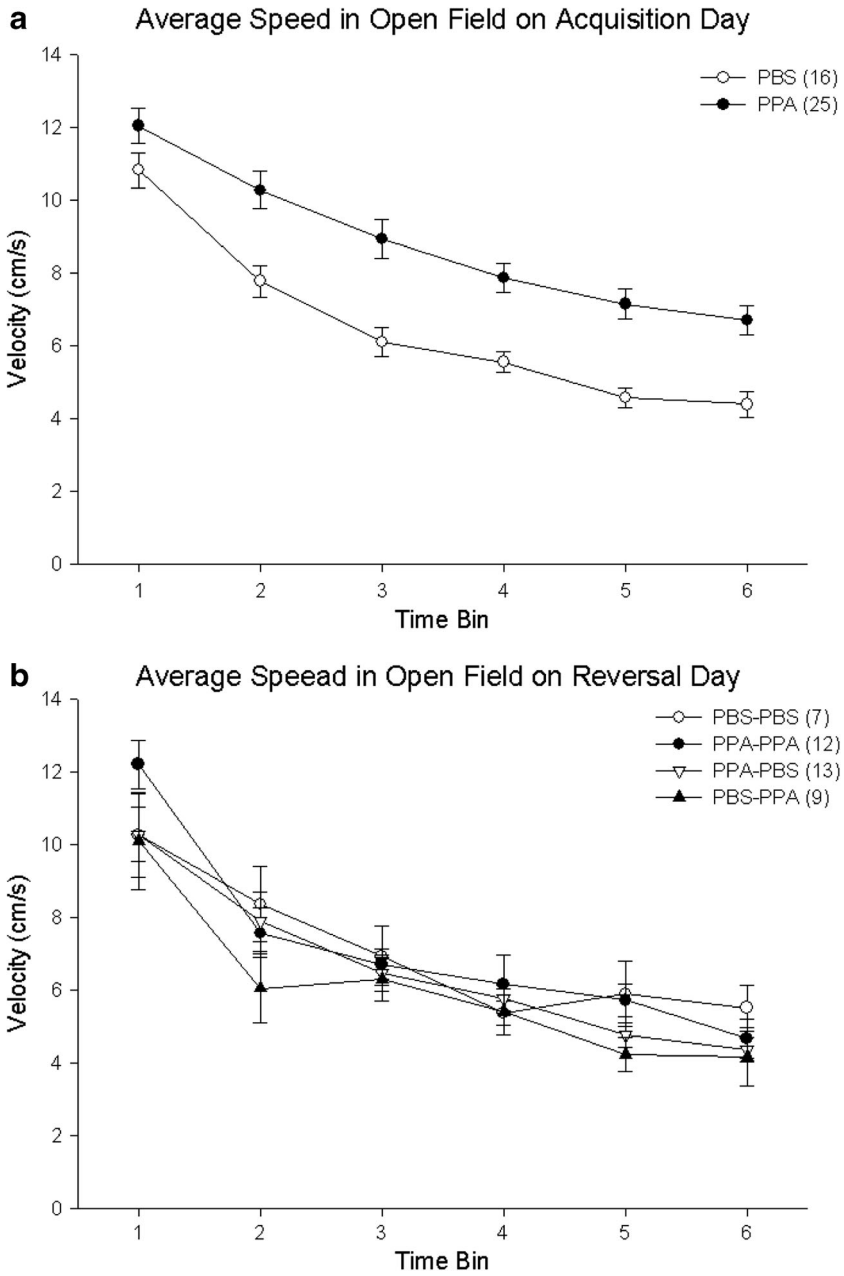

Fig. 2 Average speed (cm/s) in the open field apparatus on Acquisition day (**a**) and average speed on reversal day (**b**) for rats injected (ICV) with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each time bin in the 30-min session immediately following injection. Error bars represent ±SEM. PPA-treated rats exhibited significantly greater average speed than PBS controls on Acquisition day. There were no significant group differences on reversal day

On reversal day, as shown in Fig. 5b, all treatment groups exhibited decreased distances traveled across trials, $F(9, 189) = 6.239$, $p < .001$. However, groups receiving PPA on reversal day continued to travel larger distances than controls, $F(3, 21) = 4.230$, $p < .05$. There was no significant interaction between trial and drug treatment, $F(27, 189) = .800$, $p = .276$, indicating that the improvement across trials was similar across drug groups.

Time Spent in the Periphery of the Pool

During acquisition training, all treatment groups exhibited decreased time spent in the periphery of the pool as training progressed (Fig. 6a). However, PPA-treated rats continued to spend more time in the periphery than the PBS controls. These impressions were confirmed by ANOVA, with significant main effects being found for both trial, $F(9, 189) = 13.793$, $p < .001$, and drug treatment $F(1, 21) = 10.664$, $p < .01$. There was also a significant interaction between trial and drug treatment, $F(9, 189) = 3.949$, $p < .01$, indicating that the decrease in time spent in the periphery of the pool across trials was less for the PPA-treated rats than for controls. PPA rats continued to spend more time in the periphery over trial blocks 2, 3, 4, and 5.

On reversal day, as seen in Fig. 6b, all treatment groups exhibited decreased time spent in the periphery of the pool

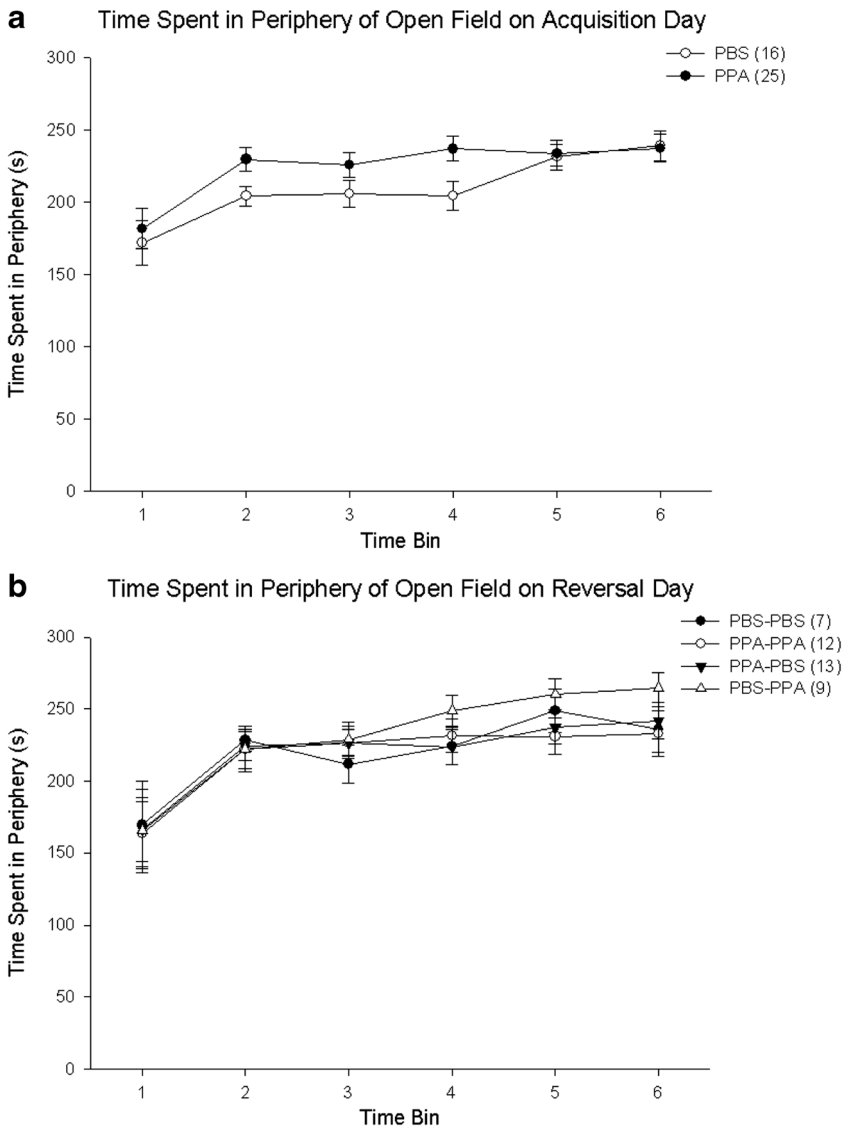

Fig. 3 Time (s) spent in the periphery of the open field on acquisition day (**a**) and on reversal day (**b**) for rats injected (ICV) with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each time bin in the 30-min session immediately following injection. Error bars represent ±SEM. There were no differences on either test day in the amount of time spent in the periphery of the open field

across trials, $F(9, 189) = 7.916$, $p < .001$. There was also a significant main effect of drug $F(3, 21) = 4.075$, $p < .05$. There was found to be no significant interaction between trial and treatment, $F(27, 189) = .884$, $p = .340$, indicating that the improvement across trials was similar across drug groups.

Number of Direct and Circle Swims

As shown in Fig. 7a, during acquisition training, the PPA-treated group exhibited fewer direct and circle swim paths than PBS controls. ANOVA confirmed this impression, revealing a significant treatment effect, $F(1, 21) = 12.588$, $p < .01$, with the PPA group displaying fewer direct and circle swims than PBS controls. As shown in Fig. 7b, during reversal training, there was no significant main effect of treatment, $F(3, 18) = 3.028$, $p = .056$.

Discussion

This study examined the lasting cognitive effects of ICV PPA loading, after a 1-week recovery period. It was found that treatment with PPA twice a day for seven consecutive days caused impairments in water maze acquisition. This was observed through longer search latencies, increases in travel distances, and fewer direct and circle swims.

Water Maze

The variables analyzed in the water maze were search latency, total distance traveled, time spent in the periphery, swim speed, number of direct and circle swims, and percent first choices to initial quadrant. On both test days, it was observed that animals were able to learn the water maze, with

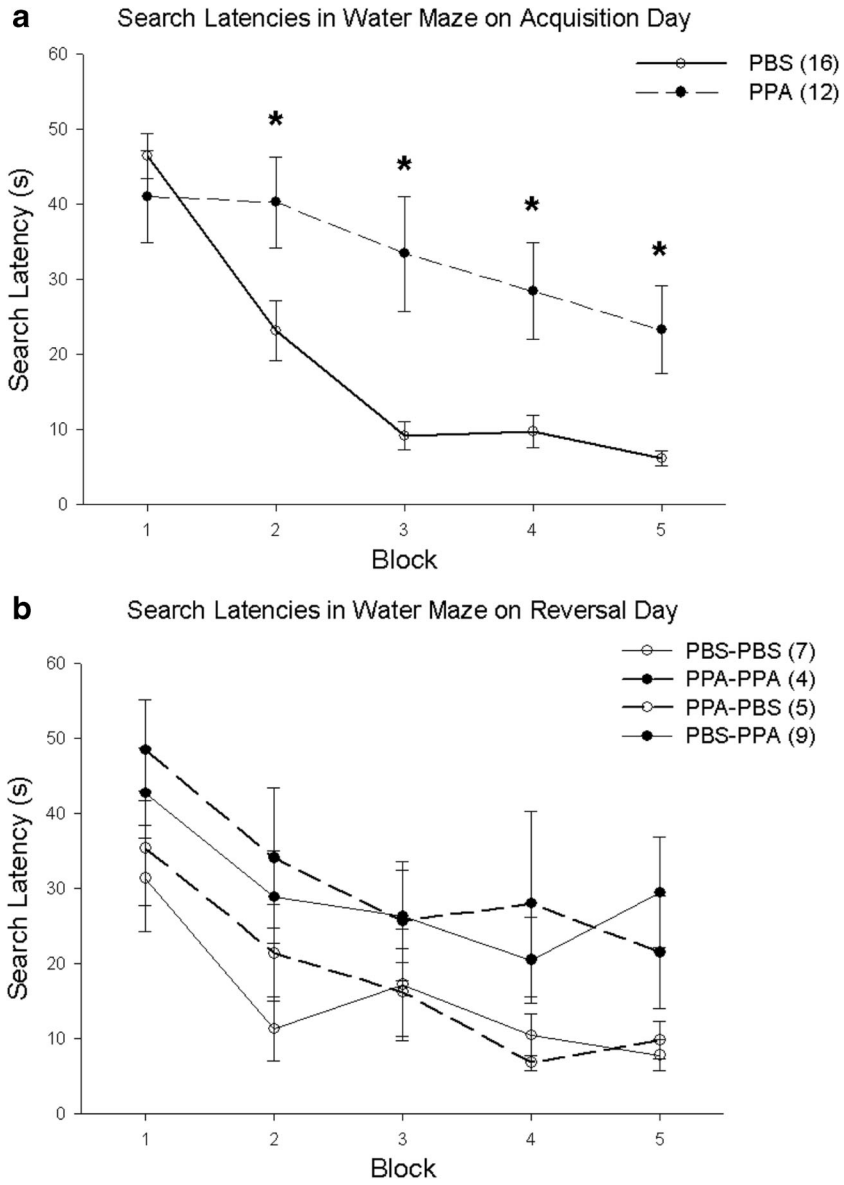

Fig. 4 Search latencies (s) to find the hidden platform on acquisition day (**a**) and on reversal day (**b**) for rats injected with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each of 10 trials immediately following injection. Error bars represent ±SEM. * $p < .05$. Search latencies of PPA-treated rats improved less across the ten trials than controls on Acquisition day, and there was also found to be a significant interaction between trial and drug treatment, with search latencies of PPA rats being significantly higher than those of PBS rats on trial blocks 2 through 5. On reversal day, there were no significant group differences in search latencies

performance on all variables improving across trials. However, animals given PPA improved less across trials than did controls.

Significant group differences were seen on all variables except for swim speed on acquisition and reversal days (data not shown) and percent first choices to initial quadrant during reversal ($F(3, 18) = .376$, *ns*). The lack of a significant group difference in swim speed suggests that there were no major motor impairments experienced by the PPA-treated rats. The finding that PPA-treated rats exhibited the same percentage of first choices to the initial platform-containing quadrant on reversal day suggests that PPA-treated rats in the present study did not show perseverative behavior as was found in the study by Shultz et al. (2009), where animals received single, spaced infusions of the compound. This could be due to the fact that animals given PPA during phase I were impaired in acquiring the maze to begin with and thus had no initial memory of the location of the platform on which to perseverate.

Rats who received PPA for 7 days prior to water maze acquisition were impaired in the maze. They were unable to learn the maze as well as controls, and this was seen in increased search latencies, larger total travel distances, more time spent in the periphery of the pool, and fewer direct and circle swims. All of these findings point towards impairments in the learning of adaptive behavioral strategies in the maze. This finding is inconsistent with what was observed by Shultz et al. (2009), where animals administered PPA showed a pattern of learning that was statistically no different than controls during water maze acquisition. Shultz et al. (2009) found that PPA-treated rats displayed a pattern of water maze activity that was the opposite of what is typically seen. Rats were not impaired in acquiring the maze, but showed a marked

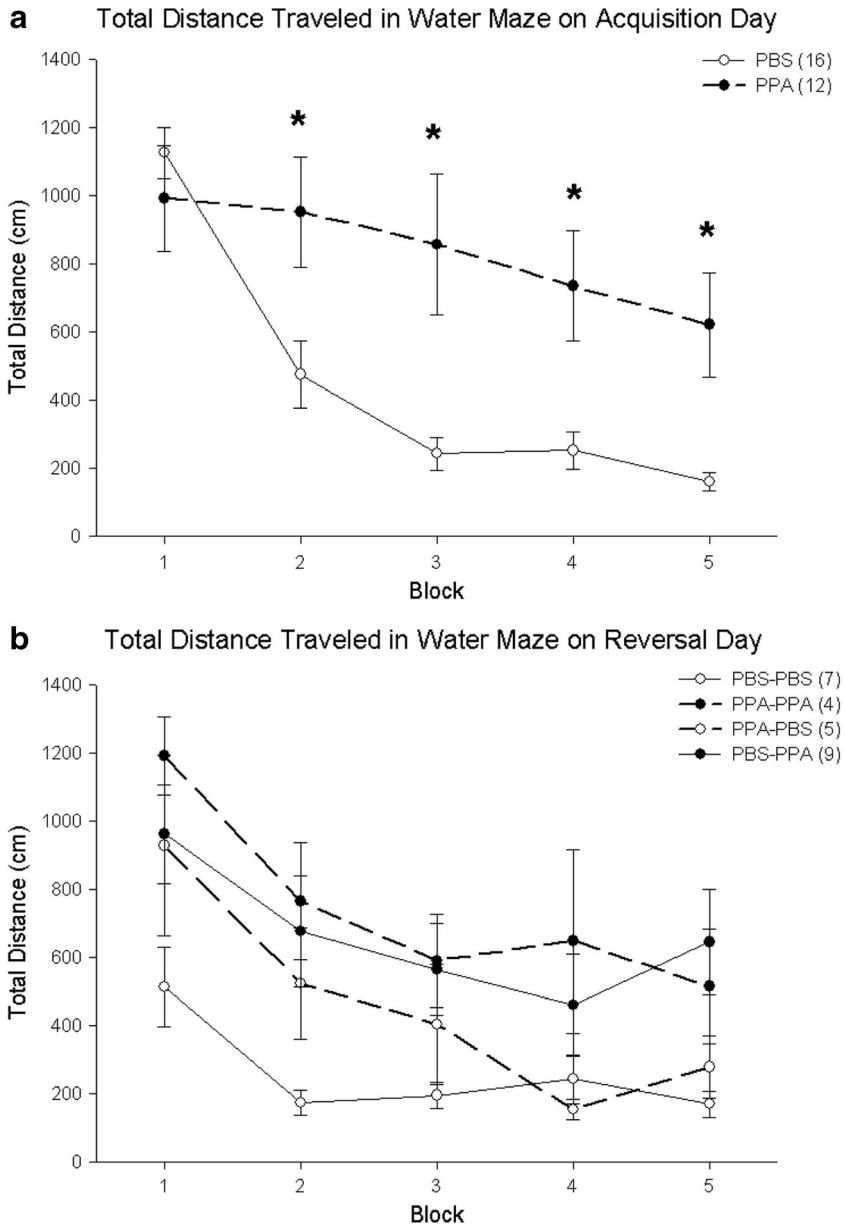

Fig. 5 Total distance (cm) traveled in the water maze on acquisition day (**a**) and on reversal day (**b**) for rats injected with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each of ten trials immediately following injection. Error bars represent ±SEM. * $p < .05$. PPA-treated rats traveled significantly greater distances on acquisition day. There was a significant interaction between trial and drug treatment, with PPA-treated rats continuing to travel significantly larger distances across trial blocks 2 through 5 than PBS rats. On reversal day, there was a main effect of drug treatment

impairment during maze reversal. This difference in findings is likely explained by the difference in methods between the present study and the study by Shultz et al. (2009). In the previous study, rats were given either three or five single (i.e., one infusion per day) ICV infusions of PPA over the course of a week prior to water maze training. In the present study, rats were tested for maze acquisition following 14 infusions of PPA administered over seven consecutive days. It has been shown that infusions of PPA can have cumulative effects in rodents. MacFabe et al. (2007) found that 5 consecutive days of single PPA infusions leads to a progressive increase in the maximum level of convulsive stage measured, as well as an increased response to a pentylenetetrazol (PTZ) treatment, which lead to the conclusion that repeated, spaced infusions of PPA have a kindling effect in rodents. Similarly, occurrences of abnormal behaviors, such as limb dystonia and hyperactivity, have been shown to increase in frequency, as well as an increase in neuroinflammation, with number of spaced infusions (MacFabe et al. 2007, 2008). Based on this, it is likely that PPA loading has a number of cumulative effects, both physiologically and cognitively, that need to be explored further.

Shultz et al. (2009) concluded, based on their findings, that rats given either three or five single infusions of PPA showed perseveration of behavior in the water maze, which was consistent with what is seen in the human disorder (Sasson et al. 2008), as well as further findings from our laboratory (MacFabe et al. 2011). The marked impairment observed in these rats during reversal training, accompanied by the increased percentage of first choices to the quadrant where the

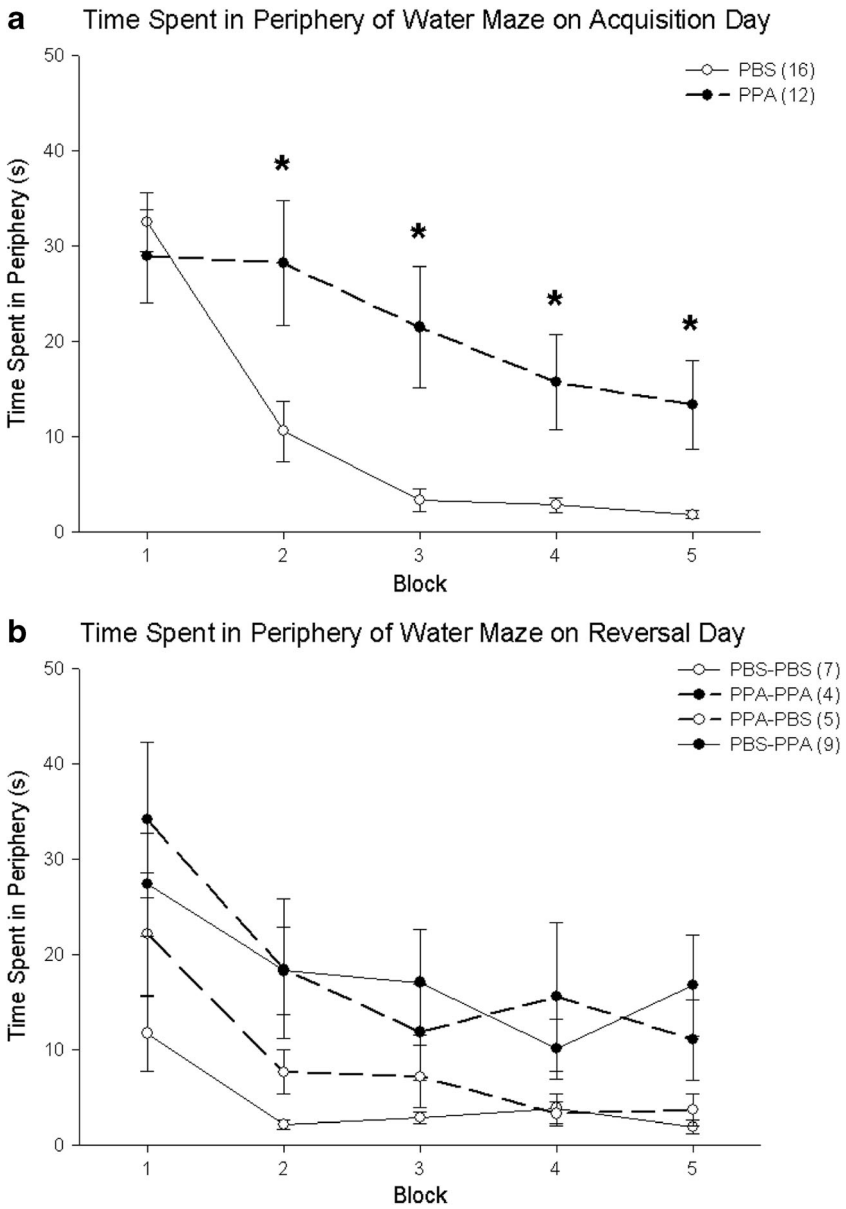

Fig. 6 Time spent in the periphery (s) of the water maze on acquisition day (**a**) and on reversal day (**b**) for rats injected with either PBS (vehicle) or PPA (0.26M). Each point represents group mean data for each of ten trials immediately following injection. Error bars represent ±SEM. * $p < .05$. The periphery represents the outer third of the pool. On acquisition day, PPA-treated rats spent more time in the periphery than controls on trial blocks 2, 3, 4, and 5. On reversal day, there was a significant main effect of drug

platform was initially located during acquisition training, suggest that the animals were having difficulty unlearning the maze. This type of perseverative behavior was not observed in the present study, with PPA treated animals showing no preference for the south-east quadrant during reversal (data not shown). Instead, what was observed was a general deficit in water maze performance.

Recovery to Baseline

One of the aims of the present study was to examine whether or not the observed, substantial cognitive deficits caused by PPA would return to baseline after administration of the compound had been discontinued for a period of time. Due to the fluctuating course of ASD symptomatology, researchers are beginning to look into the idea of "recovery" in autism; a concept that is not yet clearly defined. Helt et al. (2008) suggest that recovery should be looked at as the loss of the behavioral characteristics of ASD, bearing in mind that these children are often still not identified as "normal." To be considered "recovered," a term that is used conservatively in research, a child who had previously been diagnosed with ASD must now be learning and applying new skills which are at an appropriate age and developmental level for that child (Helt et al. 2008). For the purposes of this study, it could be said that to be considered as "recovered," a return to baseline should be achieved on all variables measured, i.e., the animal is able to reverse the maze once drug administration has been discontinued. The findings of the present study suggest that recovery to baseline after previous PPA loading (i.e., two

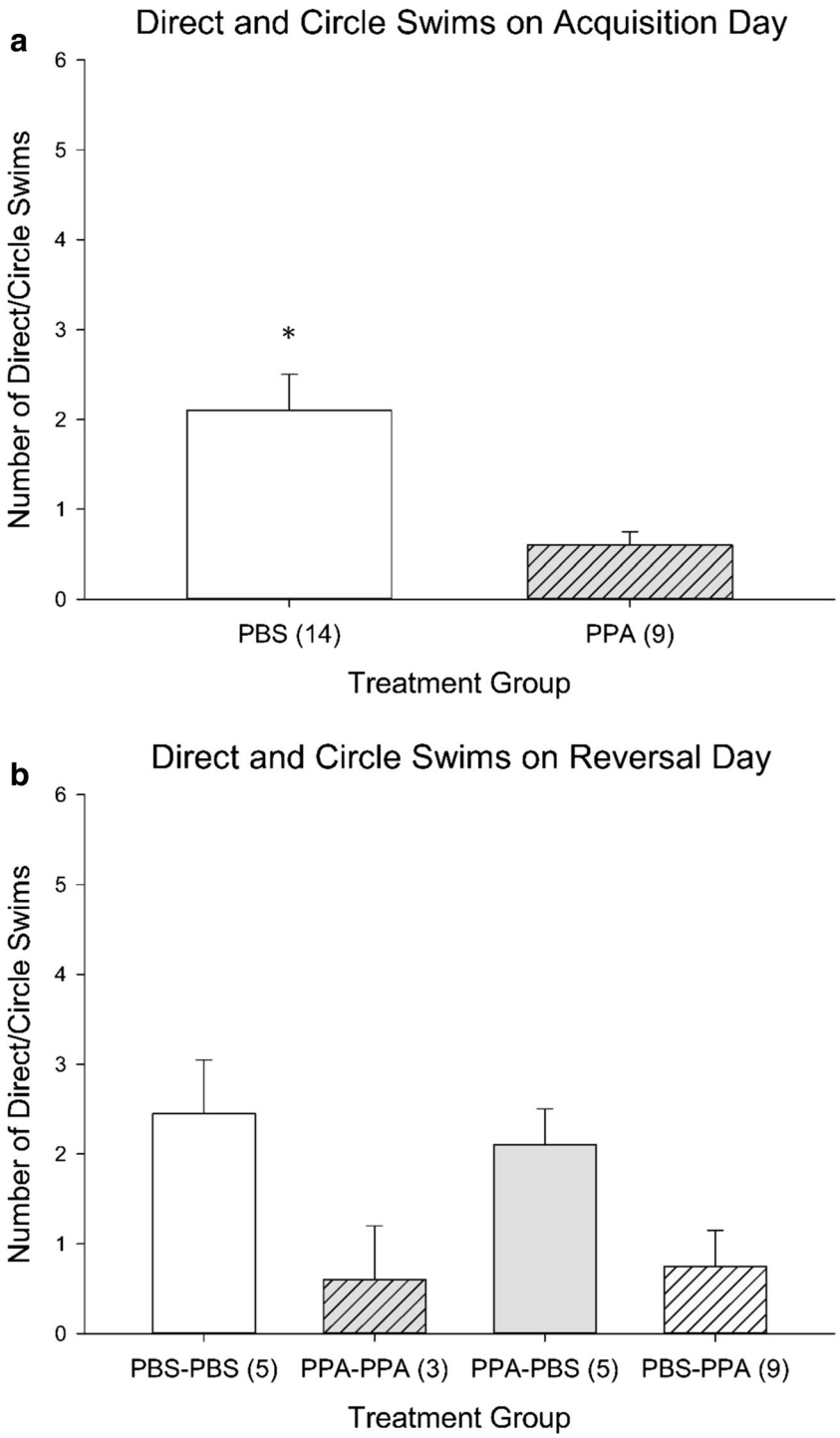

Fig. 7 Number of direct and circle swims on acquisition day (**a**) and on reversal day (**b**) for rats injected with either PBS (vehicle) or PPA (0.26M). Each bar represents group mean data immediately following injection. Error bars represent ±SEM. * $p < .05$. Rats treated with PPA exhibited significantly fewer direct and circle swims than controls. On reversal day, there were no significant group differences in the number of direct and circle swims

infusions per day for 7 days) appears possible, based on the behaviors tested in the water maze and open field, following discontinuation of the drug for a period of 7 days. Animals that received two final infusions of PPA on reversal day, regardless of which drug they received during phase I of the study, were impaired in reversing the maze compared to controls, suggesting that two infusions of PPA are enough to cause deficits in cognition. Animals in the PPA-PBS group (i.e., the "recovery" group) showed a pattern of performance on reversal day that was often at the same level as controls. The "recovery" group did show an intermediate level of performance by traveling longer distances and spending more time in the periphery of the pool than controls; however, this finding did not reach significance. This suggests that certain behaviors return to baseline, whereas others may be more permanent. Further research should be conducted to explore whether or not these intermediate deficits remain over a longer time period, or whether performance of previously PPA-loaded animals can return completely

to baseline if given sufficient time to recover. Similarly, it would be beneficial to investigate the time course of other behaviors that are relevant to ASD (i.e., social behaviors), as well as other neuropathological (e.g., CREB) and biochemical (e.g., phospholipid/acylcarnitine) markers.

There are varying levels of cognitive impairment observed in individuals with ASD. The disorder can occur with or without cognitive impairment, with some individuals experiencing profound deficits in IQ and others achieving normal levels (Dawson and Zanolli 2003; Dawson et al. 2007). In fact, the reported percentages of children with ASD who fall in the range defined as mental retardation are between about 25 and 64% (Kielinen et al. 2000). The present study modeled a more severe cognitive impairment, which has been shown to be dependent on the dose of PPA treatment (MacFabe et al. 2007; Kamen et al. 2019), with higher doses producing greater impairment. It is interesting to note that even though these animals exhibited extreme cognitive impairments in the water maze, they were still able to show improvements to baseline levels following discontinuation of PPA treatment.

Convulsive Activity

In the present study, water maze data from 16 of the 41 animals tested were removed due to convulsive activity that was observed during acquisition training in the water maze. The kindling effect of PPA has been well noted (MacFabe et al. 2007), and so the presence of convulsions throughout such a rigorous infusion schedule, such as the one used in the present study, is not surprising. Although it cannot be concluded without EEG data that some or all of the convulsive activity seen following infusions was actually seizure activity, it is a possibility. Furthermore, some seizure types (i.e., hippocampal seizures) produced by PPA are convulsive and present with movement arrest or immobility (MacFabe et al. 2007). Conversely, movement disorder has been observed in the model, which appears as convulsive behavior. This behavior, however, was accompanied by spiking in the basal ganglia, rather than cortical spiking, which is more often characteristic of seizure (MacFabe et al. 2007). If a seizure were to occur prior to or during testing, this would interfere with cognition and thus the animal's ability to learn the maze. Convulsive activity was also often observed as full-body clonus, which alone can make activities, such as swimming and climbing onto a platform very difficult. It was for this reason that data from rats that displayed convulsive activity was removed entirely from all water maze analyses. This, however, leads to a different problem, where rats that were resistant to convulsive/seizure activity were selected for, unintentionally. It is possible that the rats that were able to receive 14 infusions of PPA over the course of 7 days without displaying convulsive activity have some common characteristic that could have skewed the data presented here. Similarly, because seizure disorder is a common comorbidity of ASD (Besag 2004), eliminating this data may have resulted in the exclusion of data that may have been informative. For the purposes of this study, however, the conservative choice of leaving these data out of the behavioral analyses for water maze was deemed more suitable. Further studies should be conducted to elucidate the precise role that convulsive activity plays in the model.

Conclusions

Findings from the present study are suggestive of a return to baseline on behavioral variables measured in the water maze and open field apparatus following discontinuation of PPA treatment. The cognitive deficits caused by PPA did not appear to be the result of perseveration in the water maze, as was seen in previous work by Shultz et al. (2009). Instead, the animals appeared to be unable to acquire or reverse the maze immediately following PPA infusion. Interestingly, this severe cognitive impairment returned to baseline levels after discontinuation of PPA treatment, indicating that the cognitive deficits measured in the water maze resulting from PPA treatment are not permanent. These findings are also consistent with the hypothesis that elevated levels of PPA are putatively responsible for manifestation of an autistic behavioral phenotype.

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