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Research report

Intracerebroventricular injections of the enteric bacterial metabolic product propionic acid impair cognition and sensorimotor ability in the Long–Evans rat: Further development of a rodent model of autism

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ABSTRACT

Propionic acid (PPA) is a dietary short chain fatty acid and a metabolic end-product of enteric bacteria. Intracerebroventricular (ICV) injections of PPA can result in brain and behavioral abnormalities in rats similar to those seen in humans suffering from autism. To evaluate cognition and sensorimotor ability in the PPA model, male Long–Evans hooded rats received ICV injection of PPA or control compounds prior to behavioral testing in water maze and beam tasks. Compared to controls, PPA-treated rats were impaired in the water maze task as indicated by an unusual pattern of mild or no impairment during spatial acquisition training, but marked impairment during spatial reversal training. PPA-treated rats were also impaired on the beam task. Neuropathological analysis from PPA-treated rats revealed an innate neuroinflammatory response. These findings add to evidence that PPA can change the brain and behavior in the laboratory rat consistent with symptoms of human autism.

1. Introduction

Autism spectrum disorders (ASD) affect approximately 1 in 166 children with symptoms including abnormal motor movements, repetitive interests, seizures, cognitive deficits, and impaired social interactions [1,2]. While much research supports a strong multigenetic basis to ASD [2], other evidence suggests that environmental, dietary, and gastrointestinal factors may also contribute to the disorder [1,3,4]. Propionic acid (PPA) is a short chain fatty acid that is endogenous to the human body as both an intermediary of fatty acid metabolism and a metabolic end-product of enteric bacteria found in the gut, and is also a common food preservative [5,6]. While most PPA is produced by carbohydrate and amino acid fermentation by enteric bacteria, PPA readily crosses the gut-blood and blood-brain barriers and can gain access to the CNS by both active and passive means [7,8], where it can cross cell membranes and accumulate within cells. PPA and related short chain fatty acids (i.e. butyrate) are capable of inducing widespread effects on CNS function, including changes in neurotransmitter release and synthesis, lipid metabolism, mitochondrial function, immune activation, and gene expression [9–11]. Of particular interest is their ability to concentrate within cells, with minor reductions in pH, thus inducing intracellular acidification [12,13]. Intracellular acidification can alter neurotransmitter release, calcium signaling, and inhibit gap junctions, potentially altering neuronal communication and behavior [14–16].

PPA may be associated with ASD. Anecdotal evidence from parents of ASD children suggests that behavioral symptoms increase when their children ingest foods such as refined wheat and dairy products that contain PPA as a food preservative [3,4]. Children undergoing valproic acid therapy for epilepsy may experience increased levels of short chain fatty acids such as PPA [17], and exposure to valproic acid during early development increases the likelihood of ASD [1]. A subset of ASD patients with gastrointestinal symptoms and behavioural regression may also have elevated levels of Clostridia, an early gut colonizer known to produce PPA and

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other short chain fatty acids [58]. Furthermore, serum studies from ASD patients have provided evidence of metabolic dysfunction, including impairments of B12, glutathione or carnitine metabolism [18], that are consistent with PPA's effect on cellular metabolism [11,19]. Therefore, we recently proposed that alteration of PPA level or metabolism might be related to some symptoms of ASD, and that administration of PPA might be a means of modeling ASD symptoms in the rat [9]. We found that ICV injections of PPA induced repetitive behaviors, hyperactivity, turning behavior, retropulsion, caudate spiking, kindled seizures, impaired social behavior, increased oxidative stress markers, and induced an innate neuroinflammatory response [9,10,20], all of which appear consistent with findings from ASD patients [21–24]. Other studies that made use of PPA or 3-nitropropionic acid (3NP) in rat models of propionic acidemia or Huntington's disease yielded brain markers or behavioral symptoms that resemble certain markers and symptoms of human ASD [25,26]. These include neuroinflammation, neurodevelopmental delay and, of particular relevance to the current study, impaired cognitive and motor function as measured on the water maze and beam tasks [21,23,24].

Here we examined the effects of PPA and control compounds on spatial cognition in the water maze [27], and sensorimotor performance on the beam task [28] using the same route of administration and doses of the compounds that were employed previously in our laboratory [9,10,20]. It was predicted that PPA would produce cognitive and sensorimotor impairments together with neuropathological changes consistent with symptoms of ASD and similar to past findings associated with PPA.

2. Methods

2.1. Subjects

Subjects were 50 adult male Long–Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada). Prior to surgery for implantation of an indwelling guide cannula for intracerebroventricular (ICV) administration of the treatments rats weighed between 200 and 250 g, were housed in pairs in standard acrylic cages (26 cm × 48 cm × 21 cm) at a controlled temperature (21 ± 1.0 °C), and were naive to all experimental procedures. After surgery rats were housed individually for 14 days to allow recovery. The light/dark cycle was a 12:12 cycle with lights on from 7:00 to 19:00 h and animals were allowed access to food and water *ad libitum*. After surgery and prior to collection of data for the current study some rats participated in a social behavior study that involved two PPA injections followed by placement in a simple open field arena for two 30-min periods, with behavior tracked by an overhead camera ([20]; see below). Behavioral test procedures for the previous and current studies were separated by at least 1 week and were in accordance with guidelines of the Canadian Council on Animal Care and approved by the University of Western Ontario Animal Use Committee.

2.2. Surgery: cannula implantation

Rats were placed in a sealed Plexiglas box into which 4% isoflurane and 2 l/min oxygen flow was introduced for anesthesia. Rats were then placed in a standard stereotaxic device equipped with a gas anesthesia nose cover to maintain anesthesia throughout surgery with 2% isoflurane and 500 ml/min oxygen flow. Under aseptic conditions rats were surgically implanted with a 23-gauge 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 [29]. 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. A removable plug sealed the guide cannula until an injection was to be made. Immediately post-surgery all rats received a subcutaneous injection of analgesic (Ketoprofin, 1 ml/kg).

2.3. Treatment groups

Rats were randomly assigned to one of five treatment groups: PPA-5 (4 μl of 0.26 M solution, $n = 11$); PPA-3 (4 μl of 0.26 M solution, $n = 9$); Sodium acetate (SA; 4 μl of 0.26 M solution, $n = 10$); Propanol (PROP; 4 μl of 0.26 M solution, $n = 10$); and Phosphate-buffered saline (PBS; 4 μl, $n = 10$). All of the rats comprising the PPA-5, SA, PROP, and PBS groups had received a total of two injections of their assigned compound followed by two, 30-min periods in a simple open field arena in a social behavior study [20]. For the current study each rat in these groups received three additional injections of their assigned compound. The PPA-3 group was included to provide a group that was naive to experimental treatment prior to the start of the current study. Thus, the PPA-3 and PPA-5 groups received a total of three and five injections of PPA, respectively, by the end of the current study. SA was used to provide a second acidic experimental treatment to test the general role of brain acidification in any effects that were observed. PROP was used to provide a treatment that was a non-acidic alcohol analogue of PPA. Doses were chosen based on previous dose-response findings from our earlier studies [9,20]. Solutions were buffered to physiological pH 7.5 before injection using hydrochloric acid or sodium hydroxide.

2.4. ICV injections

Approximately 3–4 min prior to each of the three behavioral test sessions, each rat received an injection 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 injection consisted of 4 μl of solution delivered over a period of 1 min. To ensure that the entire injection had been delivered, the injection cannula was allowed to remain in place for an additional minute before being removed. The behavioral test sessions were separated by a 1 week recovery period.

2.5. Behavioral test apparatus

2.5.1. 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) located in the south-east quadrant of the pool, during acquisition, and in the north-west quadrant, during reversal. Polypropylene beads floating on top of the water prevented the rats from seeing the hidden platform [30]. Doors, cabinets, and objects in the room provided a variety of distal cues. Behavior was recorded by a video camera mounted to the ceiling above the centre of the pool. The camera was connected to a computer and behavior was objectively analyzed by a tracking system that digitized each swim trial (*Poly-Track*, *San Diego Instruments*, San Diego, CA).

2.5.2. Beam task

Fine sensorimotor ability was evaluated using a narrow wooden beam, which was 1 m long and was rigidly suspended at each end 1 m above the floor, with soft padding on the floor underneath in case a rat fell off the beam [28]. One edge of the beam was 4 cm wide and was placed facing up for initial acclimation to the task. The other edge was 2 cm wide and was placed facing up during the actual beam task. The lights in the testing room were turned off and a halogen lamp was placed at the start end to illuminate the beam and provide incentive for the rats to walk along the beam, which led to a dark platform at the far end of the beam as a goal. These conditions provide ample incentive for rats to traverse the beam [28,31]. Experience with the water maze does not affect performance on the beam task [31].

2.6. Experimental procedure

2.6.1. Water maze

Prior to the commencement of acquisition training, rats were injected with their assigned substance. Acquisition training consisted of 10 training trials, with each trial beginning with the rat being placed gently in the pool adjacent to, and facing, the pool wall, and ending when the rat stood on the hidden platform. Each trial began at one of four pool wall start locations (north, south, east, or west), with start locations pseudo-randomly ordered to prevent sequential starts from the same location. As this resulted in start locations that varied in distance from the hidden platform, for graphic presentation of search time data in Section 3, the time to reach the platform was averaged for every block of two trials (e.g. Block 1 = (Trial 1 + Trial 2)/2). Rats that failed to reach the hidden platform within 60 s of the commencement of the trial were placed on 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, rats were run in squads of 2 so that the inter-trial interval for the 10 acquisition trials was not more than 3 min [25].

Following acquisition training, rats were housed individually for 1 week before receiving another injection and undergoing a second water maze session for reversal training. The procedures for the reversal session were identical to acquisition except that the hidden platform was now located in the opposite quadrant of the pool.

2.6.2. Beam task

Animals were housed individually for 1 week following water maze testing. Prior to beam testing, rats were given a training session with both the 4 cm edge and the 2 cm edge of the beam for acclimation to the task. Twenty-four hours later, each rat received an injection of its assigned compound. The testing session began approximately 5 min post-injection and consisted of 10 trials. A trial began with the rat being placed on the illuminated end of the beam and ended when the animal successfully reached the dark goal platform. A maximum of 60 s was allowed for each trial. As the metabolic clearance rate of PPA is approximately 18–58 min [25], the inter-trial interval for the 10 testing trials was not more than 2 min.

2.7. Behavioral analyses

2.7.1. Cognition

Search latency and direct and circle swims were used as measures of spatial place memory [32,33]. Search latency was defined as the time in seconds from release until the rat climbed onto the hidden platform. A maximum of 60 s was allowed for each trial. Direct and circle swims were measured because they represent efficient swim paths that are normally generated by control rats swimming to a fixed visible or hidden platform [34–37]. This measure has the advantage of providing data from each trial, and is not confounded by changes in swim speed. 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 [33–36]. Direct and circle swims were summed and calculated as a percentage of the total swims for each test session.

2.7.2. Sensorimotor

Swim speed in the water maze task, traverse time in the beam task, and slips and falls from the beam were used as measures of sensorimotor function. Swim speed was objectively calculated in cm/s for both acquisition and reversal sessions of the water maze task by the *Poly-track* system and software. Traverse time for the beam task was defined as the time required to traverse the beam, with a maximum allowed time of 60 s. Slips and falls were scored when a rat slipped from the beam and dangled by its paws or when a rat fell completely off the beam. Rats that fell from the beam were given a maximum time of 60 s.

In addition, the rats were closely monitored for possible convulsive behavior because past studies from our laboratory have found a kindling effect in some rats associated with repeated daily ICV injections of PPA [9], and the onset of seizures may be an issue in studies investigating cognition or sensorimotor ability if a seizure were to occur prior to or during testing [30,38].

2.8. Brain tissue preparation and histological procedures

On the day following the final injection of PPA or control compounds, animals were deeply anaesthetized with sodium pentobarbital (270 mg/ml IP) and transcardially perfused with ice cold PBS (0.1 M) followed by 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde solution and stored at 4 ± 1.0 °C for 24 h. Following the fixation period, brains were placed in an 18% sucrose solution for cryoprotection prior to sectioning (for cannula placement) or paraffin embedding (for immunohistochemical analysis). Coronal 40 μm thick brain sections along the cannula track were cut using a cryostat, and then mounted on glass slides. Sections were dehydrated with increasing concentrations of ethanol and xylenes, and stained with cresyl violet for Nissl substance to confirm cannula placement. Microscopic examination of the stained sections confirmed that all cannula placements were in the right lateral ventricle. Coronal blocks of the remaining brain regions were dehydrated and defatted by immersion in increasing concentrations of ethanol/xylenes, followed by embedding in paraffin wax for immunohistochemical analyses.

2.9. Immunohistochemical procedures

Serial 4 μm sections were obtained through the right dorsal hippocampus, including adjacent white matter of the external capsule. This anatomical site was chosen because it allowed reliable quantification of possible PPA-induced changes in both the hippocampus and in external capsule white matter, both areas known to show innate inflammatory changes in previous studies of the PPA rodent model and in autopsy cases of ASD [9,10,23]. Anti-glial fibrillary acidic protein (GFAP) (1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark), and Iba-1 (1:10,000, rabbit polyclonal, Wako, Richmond, VA) were used as markers for reactive astrogliosis and microglia, respectively. Anti rat CD68 (1:200 monoclonal, Serotec, Oxford, U.K.) was also used to stain for a sub population of activated microglia principally derived from peripheral macrophages [39]. Tissue sections were mounted on glass slides (SurgiPath, Canada) and dried overnight at 37 ± 1.0 °C. Sections were deparaffinized and rehydrated using standard immunohistochemical procedures for antigen recovery [40]. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide in distilled water solution for 5 min. For antigen recovery, sections were immersed in boiling 0.21% citric acid buffer (pH 6.0) for 30 min in a 1250 W microwave oven. Slides were counterstained with Gill haematoxylin (EMD Biosciences) and rinsed with PBS for 5 min. A 10% normal horse serum in PBS solution was applied for 5 min followed by the primary antibodies for 1 h at room temperature. Following the incubation period, sections were washed with PBS and incubated with biotinylated antirabbit IgG (Vector Laboratories, Burlingame CA – BA1000) as a secondary antibody for anti-GFAP and Iba-1, and (antibody) or biotinylated anti-mouse (Vector Laboratories, Burlingame, CA – 2000) as a secondary antibody for CD68 for 30 min. Tissues were again washed with PBS and stained using the avidin–biotin complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA – PK6100) for 30 min at room temperature. Following incubation, slides were washed with PBS and 3,3-diaminobenzidine DAB chromagen (Sigma – D8001) was applied for 5 min. After final rinsing slides were dehydrated, cleared, and coverslipped.

2.10. Immunohistochemistry quantification

Using a standard light microscope, eight non-overlapping digital photomicrographs (area = 160,000 μm^2), spanning the pyramidal cell layer of the hippocampus (CA1 to CA2; CA3 to hilus of the dentate gyrus) at the level of the *stratum oriens* to *stratum radiatum*, ipsilateral to the cannula placement, were captured at 250× magnification. From the same section of tissue, an additional seven digital images (area = 160,000 μm^2) of the white matter of the external capsule, dorsal and adjacent to the hippocampus, were also captured sequentially starting at the corpus callosum and ending at the lateral ventricle. A total of 15 digital photomicrographs were taken from the brain of 16 randomly selected animals (9 PBS, 7 PPA). Photomicrographs were captured under fixed microscope illumination settings and exposure times to ensure consistent image quality across all pictures. Due to the diffuse nature of GFAP and Iba-1 staining, the 'area stained' function within ImagePro Plus software was used as a semi quantitative index of immunoreactivity. This function sums the immunopositive area within a digital image to provide a total immunopositive area per image in μm^2. A standard set of color recognition criteria was created for each antibody to counter the effects of variance in the intensity of DAB labeling. To quantify CD68 staining, immunopositive nucleated cells were manually counted by technologists blinded to the treatment group. Data from images were summed on a per-region basis to yield totals for both each hippocampal region (CA1–CA2, CA3–dentate gyrus) (eight images summed per rat) and white matter (seven images summed per rat [41]).

2.11. Statistical analyses

Search latency and traverse time were analyzed by SPSS using mixed design analysis of variance (ANOVA) with treatment as the between-subjects factor and trial as the within-subjects factor. Simple effects *F*-tests were carried out when appropriate. One-way ANOVAs, with treatment as the between-subjects factor, were used to analyze direct and circle swims, swim speed, slips and falls, and immunohistochemistry analysis. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate.

3. Results

3.1. Water maze

3.1.1. Search latency

As shown in Fig. 1A, during acquisition training all treatment groups exhibited decreased search times as training progressed. However, performance of the SA-treated rats improved less than the PROP and PBS controls. These impressions were confirmed by ANOVA, with significant main effects being found for both trial ($F(9, 405) = 11.920$, $p < .001$) and treatment ($F(4, 45) = 2.755$, $p < .05$). Post hoc tests indicated that SA-treated rats exhibited longer search latencies compared to the PROP and PBS control groups ($p < .05$).

As shown in Fig. 1B, during reversal training all treatment groups exhibited decreased search times as training progressed. However, the PPA-5, PPA-3, and SA groups improved less than the PROP and PBS control groups. ANOVA confirmed these impressions with significant main effects of both trial ($F(9, 405) = 13.943$, $p < .001$) and treatment ($F(4, 45) = 5.809$, $p < .001$). Post hoc tests revealed that the PPA-5, PPA-3, and SA groups exhibited significantly longer search latencies compared to PBS- and PROP-treated groups ($p < .05$).

3.1.2. Percent direct and circle swims

As shown in Fig. 2A, during acquisition training the PPA-5 and SA groups exhibited fewer direct and circle swim paths than PBS and PROP controls. ANOVA confirmed these impressions, revealing a significant treatment effect ($F(4, 45) = 3.874$, $p < .01$), with the PPA-5 and SA groups displaying fewer direct and circle swims than PBS and PROP controls ($p < .05$).

As shown in Fig. 2B, during reversal training the PPA-5, PPA-3, and SA groups exhibited fewer direct and circle swims than the PROP and PBS control groups. ANOVA confirmed these impressions, finding a significant treatment effect ($F(4, 45) = 3.533$, $p < .05$) with the PPA-5, PPA-3, and SA groups exhibiting fewer direct and circle swims than PROP- and PBS-treated controls ($p < .05$).

3.1.3. Swim speeds

As seen in Fig. 3, all groups displayed similar swim speeds during both acquisition (Fig. 3A) and reversal (Fig. 3B) training sessions.

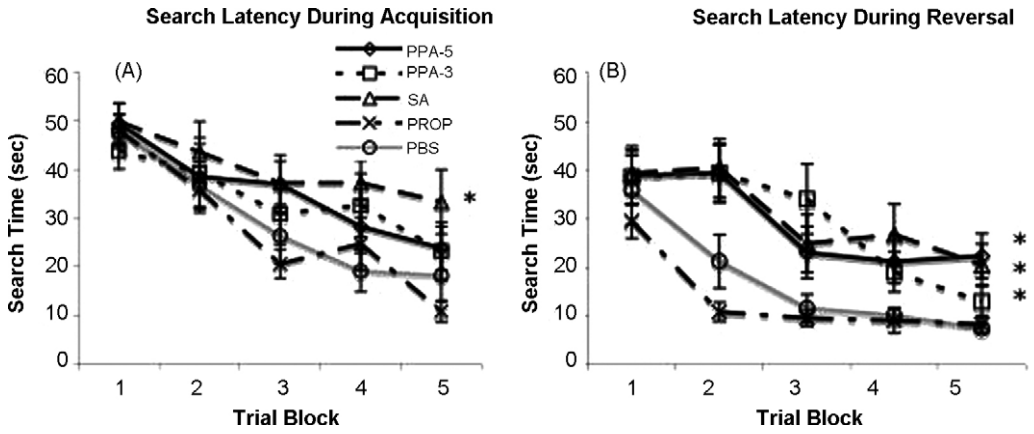

Fig. 1. (A) Search latency (s) during acquisition training of the water maze. (B) Search latency (s) during the reversal training of the water maze. Data points represent means of data collected for every block of two trials, and error bars represent ±SEM.* = different from PBS control group ($p < .05$). For additional statistical details see Section 3.

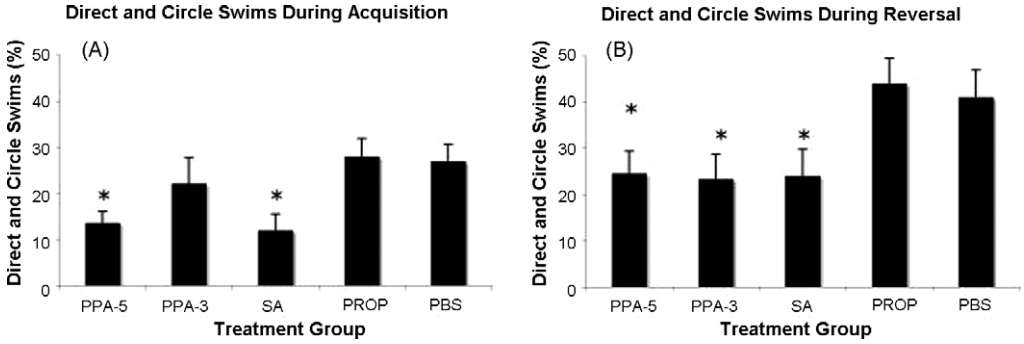

Fig. 2. (A) Summed direct and circle swims (%) during acquisition training of the water maze. (B) Summed direct and circle swims (%) during reversal training of the water maze. Histogram bars represent means of data collected during the 10 water maze trials, and error bars represent ±SEM. * = different from PBS control group ($p < .05$). For additional statistical details see Section 3.

One-way ANOVAs confirmed these impressions, with no significant treatment effect for acquisition training ($F(4, 45) = 1.427$, n.s.) or reversal training ($F(4, 45) = .402$, n.s.).

No convulsive behavior occurred immediately before or during water maze testing.

3.1.4. *Percent first choices to the acquisition quadrant*

In order to further evaluate the apparent perseverative effects of PPA during reversal training, objective evaluation of percent first choices to the initial (south-east) hidden platform quadrant made during reversal training, as described by Whishaw and Tomie [42], was carried out using the reversal training swim paths and the *Poly-Track* system. Thus, during reversal training a swim directly to the south-east quadrant would represent choosing the quadrant where the platform was located during acquisition training, and not its current location during reversal training (north-west). A one-way ANOVA with treatment as the between-subjects factor revealed a significant treatment effect during reversal training ($F(4, 45) = 3.427$, $p < .05$). Fisher's LSD post hoc pair-wise comparisons revealed that PPA-3 rats first entered the south-east quadrant significantly more frequently during reversal training ($M = 30.0 \pm 4.4\%$) than PBS rats ($M = 17.0 \pm 3.7\%$; $p < .05$) or PROP rats ($M = 13.0 \pm 3.3\%$; $p < .01$), while PPA-5 rats first entered the south-east quadrant significantly more frequently ($M = 26.4 \pm 2.4\%$) than PROP rats ($p < .05$).

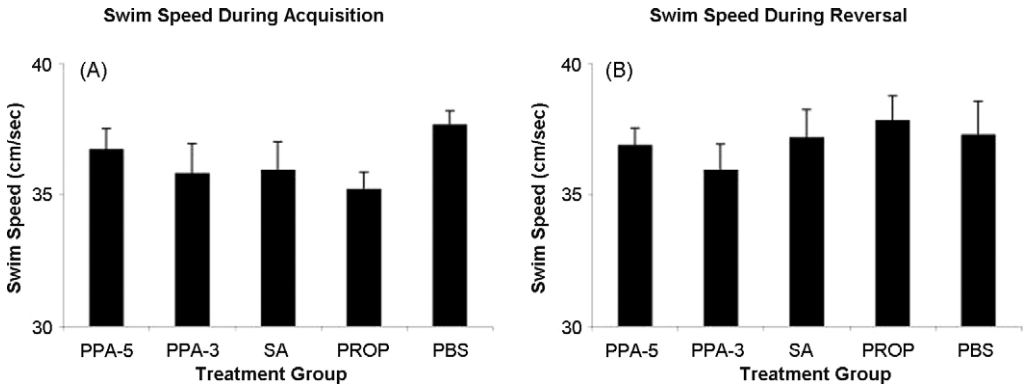

Fig. 3. (A) Swim speeds (cm/s) during acquisition training of the water maze. (B) Swim speeds (cm/s) during reversal training of the water maze. Histogram bars represent means of data collected during the 10 water maze trials, and error bars represent ±SEM. No significant differences were found between groups.

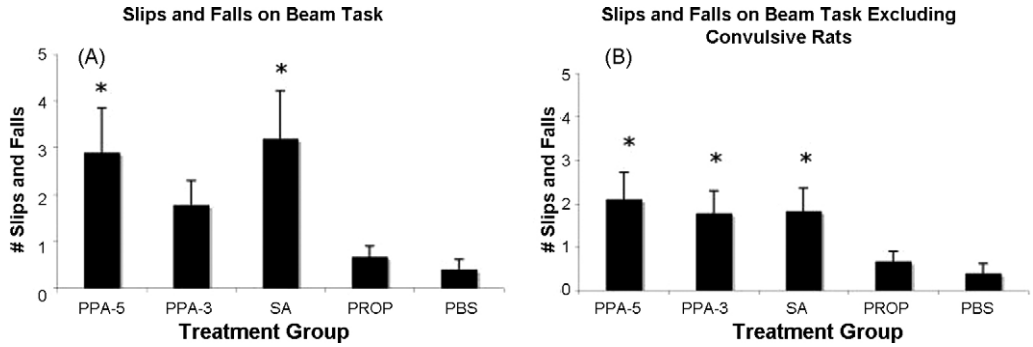

Fig. 4. (A) Mean number of slips and falls during beam task testing. (B) Mean number of slips and falls during beam task testing excluding convulsive rats. Histogram bars represent means of data collected during the 10 beam task trials, and error bars represent ±SEM.* = different from PBS control group ($p < .05$). For additional statistical details see Section 3.

Post hoc comparisons also found a non-significant trend in which PPA-5 rats tended to first enter the south-east quadrant more than PBS rats ($p = .072$).

3.2. Beam task

As shown in Fig. 4A, during beam testing the PPA-5 and SA groups displayed more slips and falls than the PROP and PBS control groups. ANOVA confirmed these impressions as a significant treatment effect was found ($F(4, 42) = 3.462$, $p < .05$), with post hoc tests revealing that the PPA-5 and SA groups displayed more slips and falls compared to PROP- and PBS-treated controls ($p < .05$).

During beam testing a total of four rats displayed convulsive behavior. To examine the behavior of rats that did not convulse a further analysis was completed with the convulsive rats excluded (PPA-5, one rat excluded; SA, three rats excluded). ANOVA using data from the non-convulsive rats indicated that a significant treatment effect remained for the slips and falls measure ($F(4, 38) = 3.191$, $p < .05$) with post hoc tests revealing that the PPA-5, PPA-3, and SA groups displayed more slips and falls than PBS controls ($p < .05$; see Fig. 4B). No significant effects were found concerning traverse times ($p > .05$; data not shown).

3.3. Neuropathology

Some tissue sections were omitted due to staining artifact or loss of tissue. Immunohistochemical results are summarized in Fig. 5. Analysis of brain tissue from PPA-treated rats showed increased GFAP immunoreactivity in the CA3–dentate gyrus ($F(1, 14) = 12.56$, $p < .01$) and CA1–CA2 ($F(1, 14) = 4.76$, $p < .05$) regions of the ipsilateral dorsal hippocampus, and external capsule ($F(1, 14) = 21.83$, $p < .001$, total mean area, $N = 9$ for PBS, $N = 7$ for PPA). There were no significant increases in total Iba-1 staining (total mean area, $N = 8$ for PBS, $N = 7$ for PPA). However, there was a statistically significant increase in CD68 labeled activated microglia in the CA3–dentate gyrus region ($F(1, 10) = 11.27$, $p < .01$) and external capsule white matter ($F(1, 10) = 15.90$, $p < .01$) of PPA-treated rats ($N = 6$ for PBS, $N = 6$ for PPA). Qualitatively there was more intense staining of all neuroinflammatory markers in external capsule white matter of PPA-treated rats compared to PBS controls, particularly in perivascular regions.

To evaluate whether two previous injections of PPA affected the PPA-5 group, statistical analyses comparing neuropathology findings of PPA-3 and PPA-5 groups were performed. GFAP immunoreactivity (mean ± SE) was greater in CA3–dentate gyrus of PPA-5 rats ($20118.89 \pm 5671.76\ \mu m^2$) than PPA-3 rats ($2481.85 \pm 1285.91\ \mu m^2$; $F(2, 22) = 11.53$, $p < .001$). GFAP immunoreactivity was also greater in external capsule white matter of PPA-5 rats ($35358.14 \pm 7769.59\ \mu m^2$) than PPA-3 rats ($4133.04 \pm 2980.52\ \mu m^2$; $F(2, 22) = 17.18$, $p < .01$). There were no significant differences between PPA-5 and PPA-3 groups in GFAP (CA1–CA2), Iba-1 or CD68 staining ($p > .05$).

4. Discussion

Our results indicate that PPA produced both cognitive and sensorimotor impairments in the adult rat. However, the particular pattern of water maze results seen in the PPA groups is unusual. Water maze studies typically report that spatial reversal learning occurs more quickly than initial spatial learning [27,36,42]. In contrast, our PPA-treated groups displayed the opposite pattern, with mild or no impairment during acquisition training, but marked impairment during reversal training. This pattern of performance is suggestive of perseveration of behavior, an outcome that bears resemblance to symptoms of ASD. The results also indicate that PPA produced a sensorimotor impairment on the beam task, which suggests that PPA-induced sensorimotor impairment might have been a factor in the PPA-induced water maze impairment.

4.1. Nature of the cognitive impairment

The pattern of behavioral results is unusual in that the PPA-treated rats had little or no difficulty in acquiring the task, but were consistently impaired in reversing the initially learned place response. The only impairment seen in the PPA groups during acquisition was in the direct and circle swim measure for the PPA-5 group, which did not differ from the PBS control group in search time. The PPA-3 group did not differ from the PBS control group on any water maze measure during acquisition training. In contrast, both PPA groups were significantly impaired on all water maze measures during reversal training. For example, during reversal training mean search times for both PPA groups were approximately twice the mean search times of the PBS controls. Similarly, the proportion of direct and circle swims of the PPA groups was little more than half the proportion of direct and circle swims of the PBS controls, and both PPA groups displayed a significantly greater frequency of first quadrant choices to the acquisition (south-east) quadrant.

The fact that there was mild or no impairment during acquisition training, but marked impairment during reversal training is unexpected with the water maze task, and has seldom been found. The typical outcome with control rats is that spatial reversal learning occurs more quickly than initial spatial learning [27,36,42], and this same outcome is frequently found with animals given various experimental treatments [43,44]. As evaluated with the water maze task, the impairing effect of PPA appears to be largely selective for spatial reversal learning. More specifically, the finding that PPA-treated rats were more likely to first visit the initial acquisition

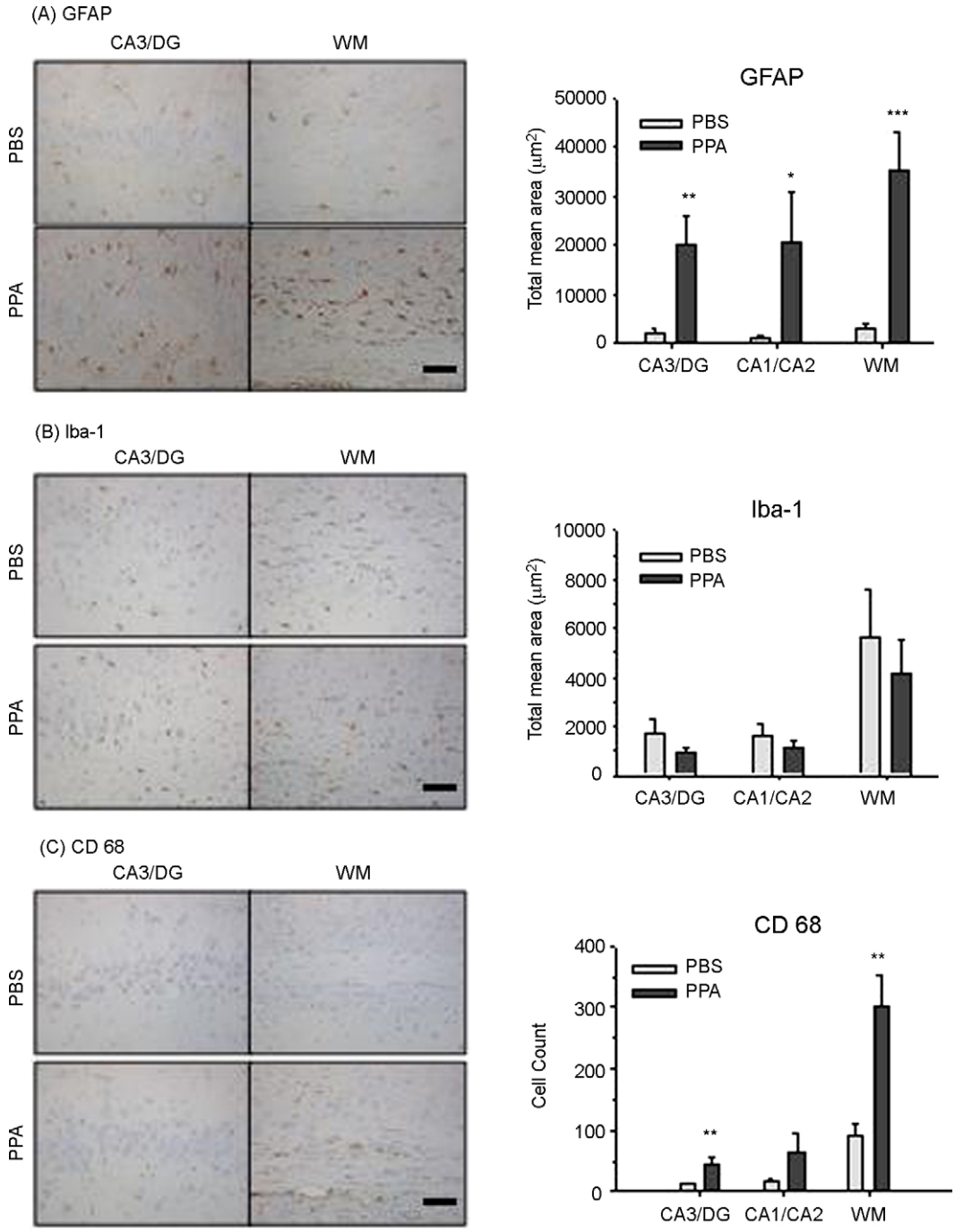

Fig. 5. Representative photomicrographs (avidin–biotin/diaminobenzidine immunohistochemistry, haematoxylin counterstain) and immunodensity quantification of dorsal hippocampus (ipsilateral CA3–dentate gyrus, CA1/CA2 regions) and adjacent external capsule white matter (WM) of rats receiving ipsilateral ICV injections of PBS or PPA (once weekly ×5 weeks). PPA produces significant increases in reactive astrogliosis (A: GFAP immunoreactivity) and macrophage derived activated microglia (C: CD 68) but not total microglia (B: Iba-1 immunoreactivity). Bars represent mean value (±SEM) for PBS and PPA-treated groups $^{*}p < .01$, $^{**}p < .001$, $^{***}p < .0001$ (original magnification 25×, scale bar represents 100 μm).

(south-east) quadrant during reversal training compared to PBS and PROP controls suggests that the cause of this spatial reversal impairment may be due to perseveration of responding to the original platform location.

The fact that some groups were impaired on the beam task, indicating sensorimotor disturbances, suggests that motor impairments need to be considered in interpreting the water maze results. Observation of the rats during swimming indicated no gross difficulty in swimming or climbing onto the hidden platform. Similarly, there were no group differences in swim speed, and all rats were able to navigate in the water and use the hidden platform as refuge. These observations and results are consistent with the generally good performance of the PPA-treated rats during water maze acquisition training. However, our previous findings with PPA administered intraventricularly suggests that there is greater immunohistochemical evidence of neuropathology in hippocampal areas CA3, dentate gyrus, and adjacent external capsule white matter with increased numbers of PPA administrations [9,10]. Given that water maze reversal training and beam task testing occurred near the end of the experiment, it is possible that PPA-induced neu-

ropathology played a role in both water maze reversal impairments and beam task impairments.

Although findings from our laboratory have previously reported a kindling effect associated with repeated daily ICV injections of PPA [9], seizure activity did not appear to influence the water maze findings, as no rats displayed convulsive behavior prior to or during water maze testing. Although seizure activity may have influenced beam task performance in a small number of rats that displayed convulsive behavior, sensorimotor impairments were found in non-convulsed rats given PPA and SA. This suggests that the presence of convulsive behavior was not required for the sensorimotor impairments seen in rats given PPA or SA. It seems likely that the repeated daily PPA injection schedule used in our previous study was important for producing seizures [9], whereas PPA injections spaced 1 week apart, as in the present study, present a low risk for seizures.

Our finding of cognitive impairments in adult rats given PPA is consistent with results reported by Pettenuzzo et al. [45] in a study of a rat model of propionic acidemia that involved chronic subcutaneous injections of PPA during early development, followed by water maze testing in adulthood. The present finding of sensorimotor impairments in PPA-treated rats is also consistent with past beam task findings using a derivative of PPA, the mitochondrial toxin 3NP [26]. Utilizing a rat model of Huntington's disease, these researchers discovered that rats receiving IP injections of 3NP completed fewer trials and made more footslips on the beam task when compared to PBS controls.

There are a number of ways in which PPA might affect brain function to cause the behavioral impairments reported here. PPA has been shown to induce an innate neuroinflammatory response, consisting of increased astrogliosis and microglia activation in the hippocampal region and external capsule white matter [9]. Similar neuroinflammatory responses have also been found in Parkinson's, Alzheimer's disease, and AIDS dementia complex with some evidence suggesting that this response may impair normal brain function [23,46–48]. We previously found [20] that two injections of PPA resulted in reactive astrogliosis, suggesting a neuroinflammatory response. Given that all rats, except those from the PPA-3 group, partook in this previous study [20] it is important to consider whether the previous two injections of PPA contributed to the findings of the current study. As PBS and PROP rats were not impaired throughout the previous study it is unlikely that the experience of prior open field social behavior testing affected the outcome of the current water maze study. However, the finding that PPA-5 rats, but not PPA-3 rats, were impaired on the direct and circle swim measure during acquisition in the current study suggests that the previous injections of PPA did affect water maze performance. The finding of greater GFAP reactivity in CA3–dentate gyrus and external capsule white matter in PPA-5 rats compared to PPA-3 rats suggests that neuroinflammation in these brain areas may have played a role in the cognitive impairments found in the current study.

In this study, comparatively brief exposure to PPA also induced reactive astrocytic and activated microglial changes in the same brain areas, but these changes were not as robust as those observed with longer PPA exposures in previous studies from our laboratory [9,10]. The observed increase in microglial CD68 staining, which labels activated microglia derived from peripheral macrophages, coupled with the absence of staining differences in Iba-1 staining, a marker of total microglia, is consistent with a subset of activated microglia being "recruited" from migration of peripheral macrophages into the CNS, leading to the production of inflammatory cytokines and reactive oxygen species such as hydrogen peroxide and nitric oxide [49]. This chemotactic effect is conceivable in our model and in ASD, as specific short chain fatty acid receptors exist on immune cells [50], and increased levels of macrophage chemotractant protein have been observed in the CSF of ASD patients [23].

However, it is important to note that innate neuroinflammatory processes may not solely explain the behavioral changes in the current study. PPA is also capable of inducing rapid behavioral changes following injection [9,20], potentially through the mechanism of intracellular acidification and associated alteration in serotonin levels by PPA [13]. As PPA-3 rats were not impaired during acquisition, which took place immediately following their first injection of PPA, it is unlikely that a fast-acting mechanism alone was responsible for the cognitive impairments observed. However, it is possible that the combination of a fast-acting mechanism in the presence of neuroinflammation caused the behavioral impairments.

PPA is known to reversibly inhibit mitochondrial function via the production of the cytotoxin propionyl Coenzyme A and through the sequestration of carnitine, leading to impairments in fatty acid metabolism [19]. Both of these effects could lead to a diffuse encephalopathic process due to an intercellular accumulation of PPA and other fatty acids, with resultant increases in oxidative stress. Encephalopathic processes, such as those found in the organic acidemias, are known to produce cognitive impairment and movement disorder consistent with findings in the PPA model and ASD [11]. In support of this hypothesis, we previously found that repeated ICV injections of PPA, which induce increased locomotor activity, also produce increased levels of oxidative stress markers in widespread brain regions, together with impairments in glutathione and catalase metabolism in neocortex, thalamus, basal ganglia, and cerebellum [10].

Moreover PPA and related enteric short chain fatty acids (i.e. butyric acid) are capable of inducing widespread changes in CNS gene expression, presumably through modulation of histone acetylation. Of particular interest, studies in our laboratory and via in vitro systems have shown that these short chain fatty acids can induce phosphorylation of cyclic AMP responsive element binding protein (CREB; [9,51]), a key factor in epigenetic expression of genes implicated in neuroplasticity and memory acquisition. Thus, it is possible that increased activation of CREB-dependant memory pathways following PPA administration could lead to normal memory acquisition with perseverative behaviors similar to those observed in the current study.

In addition to the cognitive deficits, PPA also increased slips and falls on the beam task in non-convulsive rats, suggesting that PPA caused sensorimotor impairments that were independent of convulsions. The sensorimotor impairments found in the current study might be related to our earlier finding that PPA-induced abnormal motor movements such as limb dystonia, snake-like posture, and retropulsion independent of behavioral convulsions [9]. Intracellular acidification might also explain the occurrence of sensorimotor impairments, as the onset of intracellular acidification is accompanied by a rapid increase of dopamine (DA) release [14,52].

4.2. Relation to ASD

The findings from the current study indicate that PPA induces cognitive and sensorimotor deficits in the rat, which appear to bear some similarity to symptoms seen in ASD. Individuals with ASD often display cognitive deficits, and ASD has a high co-morbidity rate with developmental delay [53,54]. More specific examples of these cognitive abnormalities include repetitive interests and resistance to change [53]. The unusual pattern of water maze impairment seen in the current study, in which rats treated with PPA were far more impaired during reversal training than during acquisition training, appears to be consistent with symptoms of repetitive interest and resistance to change seen in human ASD. In addition, the finding of impairment in the beam task, which requires fine motor coordination, appears to be consistent with symptoms of stereotyped movements and gait disturbances seen in human ASD [53,55]. Moreover, the proposed mechanisms underly-

ing the cognitive and fine motor deficits are also theoretically linked to ASD, as white matter neuroinflammation, serotonin alterations, oxidative stress, and mitochondrial dysfunction have been found in autistic individuals [22,23,56]. Interestingly, specific serotonin reuptake inhibitors, which have some efficacy in the treatment of ASD, can increase bicarbonate production through the activation carbonic anhydrase, an effect that could theoretically reverse the intracellular acidification by PPA [57].

In summary, direct ICV injections of PPA in adult male rats induced cognitive and sensorimotor impairments together with innate neuroinflammatory changes principally involving reactive astrogliosis and activated microglia. As PPA and SA rapidly induced similar changes in most behavioral measures, it seems possible that the ability of these compounds to induce effects caused by intracellular acidification or metabolic dysfunction may offer a plausible explanation for the behavioral deficits. The current findings of cognitive and sensorimotor deficits, and neuroinflammatory changes in PPA-treated rats are consistent with behavioral and neuropathological findings seen in ASD and, taken together with past findings from the PPA model, support the use of PPA in an animal model of the disorder. In addition, findings in this and previous studies from our laboratory offer further support that gut derived factors such as dietary or enterically produced short chain fatty acids may be plausible environmental factors which can trigger ASD or ASD related behaviors in sensitive sub populations. Further research involving administration of these compounds via various routes, and at critical neurodevelopmental time periods is needed to better understand the underlying mechanisms responsible for the behaviors induced by PPA treatment and their potential involvement in human ASD.

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