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Research report

Effects of the enteric bacterial metabolic product propionic acid on object-directed behavior, social behavior, cognition, and neuroinflammation in adolescent rats: Relevance to autism spectrum disorder

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ABSTRACT

Recent evidence suggests that a variety of environmental factors, including dietary and gastrointestinal agents, may contribute to autism spectrum disorders (ASD). Here we administered propionic acid (PPA), a short chain fatty acid that is used as a food preservative and also is a metabolic end-product of enteric bacteria in the gut, to adolescent (41 ± 4 days) male rats in a study of restricted/repetitive behavior, social behavior, and cognition. The goal was to further evaluate the effects of PPA in young rodents. PPA (4 μl of 0.26 M solution) was administered intracerebroventricularly prior to each behavioral test. Rats treated with PPA displayed restricted behavioral interest to a specific object among a group of objects, impaired social behavior, and impaired reversal in a T-maze task compared to controls given phosphate buffered saline. Immunohistochemical analysis of brain tissue from PPA rats revealed reactive astrogliosis and activated microglia, indicating an innate neuroinflammatory response.

These findings are consistent with our earlier findings of ASD-relevant behavioral and brain events in adult rats given PPA, and support further study of effects of PPA in young rodents by establishing similar effects in adolescent animals.

1. Introduction

The prevalence of autism spectrum disorders (ASD) is approximately 1 in 110 children [17]. Behavioral symptoms of ASD include restrictive and/or repetitive interests and behaviors, impaired social behavior, cognitive impairment, and convulsions, suggesting broad neurodevelopmental abnormality in ASD [7,22]. Although there is a strong genetic component to the etiology of ASD [22,43], recent research suggests that ASD can be exacerbated by a number of environmental factors in sensitive sub-populations [28]. Recent studies suggest a link between dietary factors or gastrointestinal disturbances and ASD symptoms, but the exact mechanisms by which such factors might contribute to ASD are not clear [7,29,32]. Some clinical studies have also found that a subset of ASD patients have high levels of Clostridia or Bacterioidetes in the gut, which produce propionic acid (PPA) and other fatty acids by anaerobic fermentation of dietary carbohydrates and some amino acids [26,54]. 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 endproduct of enteric gut bacteria such as clostridia and propionibacteria [4,40,58,67]. Parents of some ASD children report that ASD symptoms are exacerbated when the children crave and consume processed wheat or dairy products that contain PPA as a food preservative [29,32]. Rat models of propionic acidemia based on administration of PPA or 3-nitropropionic acid (3NP), a derivative of PPA, have revealed behavioral symptoms and brain markers consistent with human ASD, including developmental delay with cognitive impairments, and neuroinflammation [6,12,48,59]. Consistent with such effects on brain and behavior, PPA readily crosses the gut–blood and blood–brain barriers by both passive and active means [33], thus potentially gaining access to the brain where it can accumulate in cells and alter multiple neurophysiological processes, including neurotransmitter release, gene expression, mitochondrial function, immune modulation, gap junction gating, and ultimately behavior [15,35,47].

There is a need for a defined set of behavioral tasks relevant to the symptoms of ASD in animal models of the disorder (see [20,53]). Earlier studies with PPA in our laboratory found that intraventricular administration of PPA to adult rats induced repetitive

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behaviors, impairments in cognition and social behavior, and brain events including epileptiform spiking in neocortex, hippocampus and caudate nucleus, seizures with convulsions, increases in oxidative stress markers, reductions in glutathione, alterations of brain phospholipids/acylcarnitines and an innate neuroinflammatory response [35,36,51,52,57]. These outcomes appear to be consistent with ASD behavioral symptoms and brain events [6,18,20,22,59]. Adolescence is a key developmental period, with exacerbation of many ASD associated symptoms [42]. As part of a systematic investigation of PPA with young rats, this study examined the effects of PPA in adolescent rats. Data were obtained using a test of interaction with multiple objects to study restricted/repetitive behavior, a test of object vs. rat interaction to study social impairment, a T-maze test of cognition, and a measure of locomotor activity. We hypothesized that PPA treatment would increase restricted/repetitive behaviors, and impair social behavior and cognition [51,52] in adolescent rats. At the completion of the study brain tissue was examined using neuropathological markers for innate neuroinflammation [59].

2. Methods

2.1. Subjects

Long–Evans male hooded rats were obtained at age 26 days from Charles River Laboratories (Quebec, Canada) and housed in groups of 3 or 4 at 21 ± 1 °C in acrylic cages (26 cm × 48 cm × 21 cm) for 8 days for acclimation to the animal colony, with lights on from 7:00 to 19:00 h and access to food (LabDiet RMH 3000) and water *ad libitum*. Post-surgical housing was individual for 7 days to allow recovery. Procedures complied with Canadian Council on Animal Care guidelines and were approved by the University Animal Use Subcommittee.

2.2. Treatment groups

After recovery rats were randomly assigned to the PPA group ($n = 20$) or PBS (phosphate buffered saline) group ($n = 17$). Prior to each test session PPA subjects received an intracerebroventricular injection of 4 μl of 0.26 M solution PPA buffered to pH 7.5. PBS subjects received an equivalent volume of PBS. Doses and volumes were chosen based on our previous findings of dosage for producing behavioral symptoms and brain events of interest [35,36,51,52,57], and pilot data.

2.3. Surgery: cannula implantation

For surgery a rat was anaesthetized with 4% isoflurane and 2 L/min oxygen and placed in a standard stereotaxic device, with maintenance of anaesthesia. A 23-gauge guide cannula was implanted under aseptic conditions with the tip in the right lateral ventricle, as described previously [35,36,51,52]. Cannula placements were at A–P: +5.9 mm; M–L: +1.6 mm; D–V: −2.9 mm relative to skull surface, and were guided by the [50] atlas of the developing rat brain, and pilot work. A removable obturator sealed the guide cannula until an injection was to be made.

2.4. Apparatus

Object-directed behavior and novel rat vs. object tests took place in a circular open-field arena (90 cm diameter, 40 cm high). A CD camera was mounted above the arena, and the room was illuminated by conventional fluorescent lighting. The camera was connected to a computer, allowing behavior to be recorded and analyzed using the *EthoVision 3.0.15 Behavioral Monitoring and Analysis System* at a rate of 5.99 frames/s. Behavior was also video-recorded for later analysis. Various small plastic or metal child's toys approximately 5 cm × 7 cm × 8 cm could be placed in the arena to serve as novel objects. A small cage with a circular Plexiglas top and bottom and a wire mesh cylindrical wall (diameter, 18 cm; 1.0 cm wire mesh) was used to house a novel male stimulus rat of the same body weight as the experimental rat during the object vs. rat test. A group of rats obtained at the same time and from the same source as the experimental rats were housed under similar but separate conditions and served as stimulus rats in the novel rat vs. object test. No stimulus rat was used in more than 6 tests.

The T-maze task made use of a plywood maze painted gray with a shaft 100 cm long and 12 cm wide, with walls 15 cm high. The arms of the T were connected to the top of the shaft and were each 43 cm long and 15 cm wide, with walls 15 cm tall. A removable opaque partition allowed the test rat to be confined to a start box at the base of the shaft prior to each trial. Identical small metal cups were placed near the end of each arm (1 per arm) into which a food reward could be placed.

2.5. Treatments and behavioral testing

Before testing each rat received an injection of its assigned treatment directly into the right lateral ventricle via a 30-gauge injection cannula connected to a Sage syringe pump over a period of 1 min as described previously [35,36,51,52]. Testing for each rat began approximately 1 min after the injection cannula was removed and replaced by an obturator. Behavioral testing began at age 41 ± 4 days. All testing was carried out during the light phase of the light–dark cycle.

All rats were used in all behavioral tests, with PPA or PBS administered shortly before each test. Test sessions were separated by intervals of 24 or 48 h, as indicated below, based on our previous finding of an active period of PPA in brain for producing behavioral and brain electrographic changes lasting no more than 40–60 min [35]. Due to the expected convulsive effects of repeated injections of PPA [35], all rats were closely monitored for convulsive behavior and general health both during behavioral testing, and daily in the animal colony. As expected, no convulsions were observed early in behavioral testing, i.e., during the object-directed behavior test and the novel rats vs. novel object test. Convulsions occurred late in testing during the T-maze task in three rats after 4–6 PPA treatments had been given, and for this reason these rats were excluded from further T-maze testing and data analysis. No rats treated with PBS displayed convulsive behavior at any time during the study.

2.5.1. Object-directed behavior

This test assessed behavioral symptoms of restricted/repetitive interests [22] by determining whether PPA-treated rats preferentially directed their behavior toward a particular novel object among a group of novel objects, relative to PBS controls. The novel objects were three different small toys placed equidistant from each other approximately 10 cm from the wall of the open-field arena. After injection of the assigned treatment the rat was placed at the center of the arena and allowed to explore and interact with the objects for 5 min. Rats were tested one at a time and the arena and objects were cleaned with an alcohol–water solution after each test. Each rat was tested once and the same 3 objects were used for all tests, counterbalanced for position within groups.

EthoVision defined a 20 cm-diameter zone around each object and the number of entries into, and total duration (s) in each zone were determined. Video was also scored for sniffing bouts at each object by a person unaware of the group membership of each rat. A sniffing bout was scored when a rat approached an object with its snout placed within 1 cm of the object and with the vibrissae moving to indicate sniffing, and ended when the snout was withdrawn farther than 1 cm from the object.

2.5.2. Novel rat vs. novel object-directed behavior

This test was carried out 48 h after the object-directed behavior test and evaluated social behavior by asking whether PPA-treated rats preferentially direct behavior toward a novel rat or a novel object, relative to PBS controls. A novel male rat of the same body weight as the subject rat (see above), and an object not used in the previous object-directed behavior test, were placed opposite each other in the arena approximately 10 cm from the wall of the arena. The novel rat's movements were restricted by placing it in the small wire mesh cage described above. The subject rat was placed at the center of the arena midway between the novel object and the novel rat facing the bare wall of the arena, and allowed to explore for 5 min. Rats were tested one at a time, and the arena and object were cleaned with an alcohol–water solution after each rat was tested. Each rat was tested once and the same object was used for all tests, with stimulus object/rat positions counterbalanced within groups. *EthoVision* calculated the following measures for the subject rats: percent of time approaching the novel rat 1) or the novel object 2); total duration within 18 cm proximity of the novel rat 3) or the novel object 4). Measures 1) and 2) were obtained using *EthoVision* Relative Movement analysis. *EthoVision* calculated total distance (cm) moved to evaluate locomotor activity during the rat vs. object choice test.

2.5.3. T-maze acquisition and reversal

The T-maze task determined whether PPA-treated rats could acquire a T-maze task for food reward, and reverse the previously rewarded turn direction for food reward as effectively as controls. Habituation to the T-maze began 48 h after the previous test and involved each rat exploring the maze by itself on 3 consecutive sessions spaced 4–24 h apart, with 1 piece of food (1/3 of a Froot Loop cereal piece, Kellog) placed in each of the 3 sections of the maze. Rats explored until all 3 pieces of food were found and eaten. No injections were given prior to the habituation sessions.

For T-maze acquisition, each rat was randomly assigned to either a left or right turn as correct, with half of the rats in each group rewarded for left turns and half rewarded for right turns. Before each trial, the appropriate arm was baited by placing 1/3 of a Froot Loop cereal piece in the cup near the end of the arm. A trial started when the partition of the start box was lifted. If the rat entered the rewarded arm within 60 s, defined as placing both front paws into the correct arm, it was allowed to proceed to the cup and consume the reward. If it entered the incorrect arm or failed to enter the correct arm within 60 s it was removed from the maze and placed in a holding box. The intertrial interval was approximately 45 s. For reversal training the rewarded arm was the arm opposite to the acquisition arm. Two acquisition sessions and two reversal sessions were given, consisting of 20 trials per session. Sessions

were spaced 24 h apart. The maze was cleaned with an alcohol–water solution after each rat was tested. Records were kept of correct and incorrect turns. If the rat failed to enter an arm within the 60 s time limit a 'no choice' was scored; 'no choice' trials were not included in the statistical analysis.

2.5.4. Brain tissue preparation, immunohistochemical staining, and analysis

Within 24 h after behavioral testing animals were deeply anaesthetized, transcardially perfused, and the brains were removed and prepared for histological analysis as described previously [35,36,51,52]. This included preparing 40 μm coronal sections mounted on glass and stained for confirmation of cannula placements and the preparation of 4 μm serial sections from blocks of brain tissue embedded in paraffin for immunohistochemistry. All cannula tips were confirmed to lie in the lateral ventricle. To examine whether PPA produced neuroinflammatory changes [59,63], immunohistochemical analysis included 1) anti-glial fibrillary acidic protein (GFAP) (1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark), a marker for astroglia, and 2) anti-rat CD68 antigen (1:200, monoclonal, Serotec, Oxford, UK) [35,36,51,52]. Details were as described in [35].

Brain regions examined in 4 μm serial sections were the dorsal hippocampus and adjacent white matter of the external capsule in the hemisphere ipsilateral to the cannula placement. These areas were chosen because of their close proximity to the administration site of PPA or PBS vehicle, the cytoarchitectonics of the hippocampus that allow for reliable quantification of possible PPA-induced changes, the known role of the hippocampus in experimental kindling, human seizure disorder, and ASD, and to allow analysis of inflammatory changes in white matter of the external capsule [59].

2.5.5. Immunohistochemistry quantification

Using fixed light microscopic illumination settings and exposure times to ensure consistent image quality across all images, eight non-overlapping digital photomicrographs (area = 160,000 μm^2) spanning the pyramidal cell layer of the hippocampus (CA1–CA2) and stratum oriens to stratum radiatum were captured at 250× from each of 12 PPA group brains, and from 8 PBS group brains. From the same sections of tissue seven additional images of external capsule white matter dorsally adjacent to the hippocampus were also captured sequentially from corpus callosum to the lateral ventricle.

To quantify immunoreactivity a standard set of color recognition criteria were created for each antibody to ensure that only DAB labelled immunopositive cells were recognized by the software, thus countering the effects of variance in the intensity of DAB labelling. Data from images were summed on a per-region basis to yield totals for both the hippocampus and white matter. Due to the diffuse nature of GFAP staining, GFAP analyses were completed by using the 'area stained' function within ImagePro Plus software, which sums the immunopositive area within an image to provide a total immunopositive area per image (μm^2). CD68 staining was restricted to the cell membranes, hence these antibodies were quantified using the 'cell count' function, which counted immunopositive cells only.

2.5.6. Statistical analysis

To determine whether administration of PPA led to restricted/repetitive interests and behaviors, data from the object-directed behavior experiment were analyzed using difference scores or percent-time scores, followed by between-group comparisons using *t*-tests. This approach was necessitated by the fact that in these two experiments the behavioral data relating to the three novel objects and the novel rat vs. novel object were not independent, thus precluding conventional analysis by analysis of variance (ANOVA). Behavioral T-maze data and immunohistochemistry data were analyzed using ANOVA with Bonferroni post hoc tests where appropriate. Analysis of total distance moved data was carried out using *t*-tests. Calculations were carried out using Number Cruncher Statistical System 4.21 and GraphPad.

3. Results

3.1. Object-directed behavior

Preliminary analysis revealed no consistency within or across groups in the specific novel object that was interacted with the most. Therefore for graphing and analysis purposes, for each rat the objects were rank ordered from 1 through 3 on each behavioral measure to indicate greater-to-less interaction/interest in the objects. Thus, Object 1 was, for each rat, the object that the rat interacted with the most, Object 2 was the object that the rat interacted with second most, and Object 3 was the object that the rat interacted with the least. Of interest was the relative decrease from Object 1 to Object 2, which would address the question whether PPA rats selectively directed behavior more toward one particular novel object among a group of novel objects, relative to controls.

Fig. 1A–C presents the number of entries into each rank-ordered object zone, the total time spent in the rank-ordered object zones, and the number of sniff bouts directed to each of the 3 objects in the arena, respectively. For statistical analysis of object zone entries and object sniff bout data, difference scores consisting of (behavioral measure for Object 1) − (behavioral measure for Object 2) were obtained for each measure for each rat. Behavioral measures for Object 2 were used instead of the average of behavioral measures for Object 2 and 3 in the calculations because they provide the more conservative comparison. Thus, the difference scores represent the preference for interaction with one particular object over the other objects in the arena. For analysis of duration in object zone data, percent of time in Zone 1, calculated as time in Zone 1/total time in Zones 1 + 2 + 3, was calculated for each rat and compared between groups.

The difference scores for number of object zone entries derived from behavioral data shown in Fig. 1A differed significantly

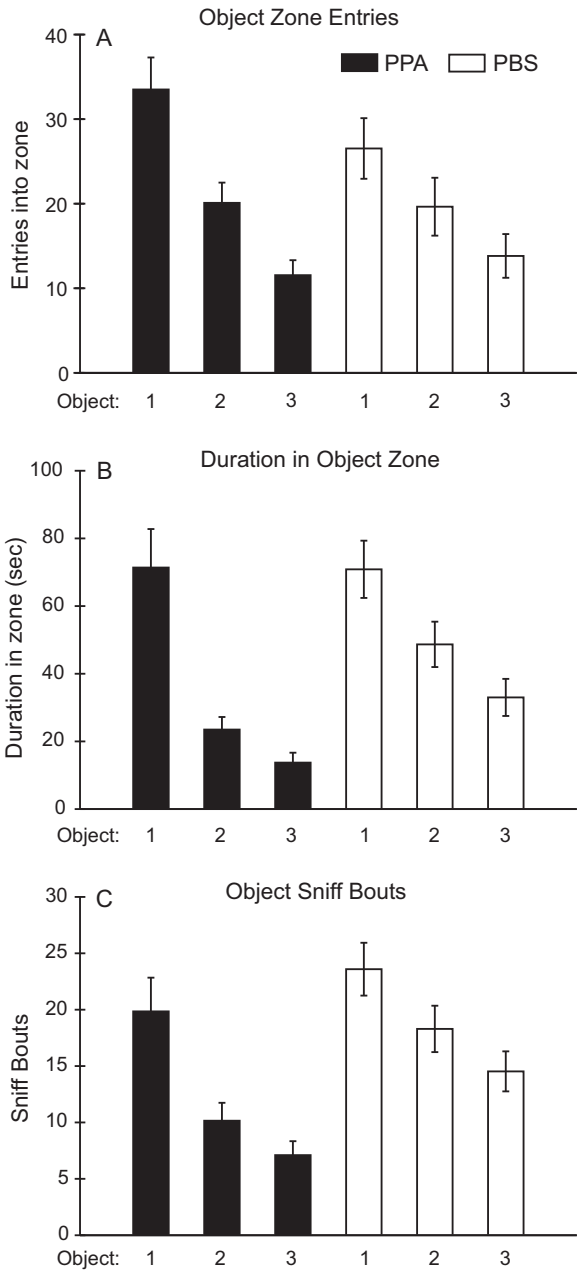

Fig. 1. Object-directed behavior group means (±SEM) of (A) number of entries into each of the 3 object zones, (B) duration of time (s) spent in the zone surrounding each of the 3 objects calculated as time in Zone 1/total time in Zones 1 + 2 + 3, (C) number of sniff bouts directed to each of the 3 objects. For further details see Section 2.

between groups, indicating that PPA rats differed in entries into the zones of objects 1 and 2 to a significantly greater extent than PBS rats (PPA group, 13.4 ± 2.7 [mean ± SEM]; PBS group, 7.0 ± 1.2; $t_{(35)} = 2.04$, $p = .049$). Percent of time in Zone 1 derived from behavioral data shown in Fig. 1B differed significantly between groups, indicating that PPA rats spent a greater percentage of time in Object Zone 1 than PBS rats (PPA group, 66.8 ± 3.4%; PBS group, 48.7 ± 2.9%; $t_{(35)} = 3.97$, $p < .0001$). There was also a nonsignificant trend for the difference scores for number of object sniff bouts derived from behavioral data shown in Fig. 1C to differ in the same direction as the other behavioral measures (PPA group, 9.7 ± 2.0; PBS group, 5.2 ± 1.2; $t = 1.80$, $p = .087$). Taken together these data suggest that PPA-treated adolescent rats show more approach behavior and more interaction with their most-preferred object than do PBS adolescent rats.

3.2. Novel rat vs. novel object-directed behavior

Fig. 2A presents the *EthoVision* movement tracks of the rat in each group whose mean distance from the novel rat was closest to the mean value of its group. Difference scores for percent time approaching the novel rat or object (time approaching the novel rat or novel object/total test session time) derived from behavioral data shown in Fig. 2B differed significantly between groups (PPA group, 17.3 ± 3.0%; PBS group, 29.8 ± 1.2%; $t_{(35)} = 3.4$, $p = .0018$). Difference scores for duration in close proximity derived from behavioral data shown in Fig. 2C also differed significantly between groups (PPA group, 112.8 ± 22.0 s; PBS group, 178.4 ± 9.2 s; $t_{(35)} = 2.6$, $p = .013$). These outcomes suggest that PPA rats show less approach behavior and remain close to the novel rat less than PBS rats.

3.3. Total distance moved

Results of the total distance moved analysis derived from *EthoVision* locomotor activity data obtained during the novel rat vs. novel object test indicated no significant difference between the groups ($t_{(35)} = 1.5$, $p = .133$; data not shown).

3.4. T-maze task

A total of 14 rats from the PPA group and 13 rats from the PBS group completed T-maze testing. The remainder were removed from the analysis because of a blocked cannula or a convulsion during testing [35]. A mean of 8.8 ± 1.7 no-choice trials occurred during the 80 trials given each rat. Results of the T-maze task are shown in Fig. 3. Separate mixed design ANOVAs were carried out on the Acquisition and Reversal phases of the task, due to the common view that these phases comprise different tasks that make use of different brain mechanisms [19,52]. ANOVA of the Acquisition data with one between group factor (PPA treatment, PBS treatment) and one within group factor (Day 1, Day 2) revealed a significant effect of day ($F(1,25) = 10.9$, $p = .002$), but no significant treatment or interaction effects ($p = .262$ and $p = .542$ respectively), indicating that both groups acquired the task. ANOVA of the Reversal data with one between group factor (PPA treatment, PBS treatment) and one within group factor (Day 1, Day 2) revealed significant effects of treatment ($F(1,25) = 5.2$, $p = .028$) and day ($F(1,25) = 46.5$, $p < .0001$) but no interaction effect ($p = .113$). Bonferroni post-tests indicated that percent correct turns increased for both groups from Day 3 to Day 4 ($p < .05$), that the PPA group achieved fewer percent correct turns on Day 4 than the PBS group ($p < .05$), but that the groups did not differ on Day 3 ($p > .05$). These results suggest that both groups learned during Reversal training, but that the PPA group exhibited impaired reversal learning at the end of Reversal training. Based on the fact that the performance of the PPA group approached 70% correct on the last day of Acquisition but returned to chance levels (approximately 50% correct) on the first day of Reversal (see Fig. 3), a supplementary ANOVA was carried out using data from both groups to evaluate any change in performance between the last day of Acquisition and the first day of Reversal. This analysis revealed a significant effect of day ($F(1,25) = 12.6$, $p = .0017$) and a significant treatment by day interaction ($F(1,25) = 6.7$, $p = .015$), but no effect of treatment ($p = .648$). The data in Fig. 3 suggest that the basis for the interaction is the large decrease in the percent correct score of the PPA group from the last day of Acquisition to the first day of Reversal, indicating impairment in reversal learning in the PPA group.

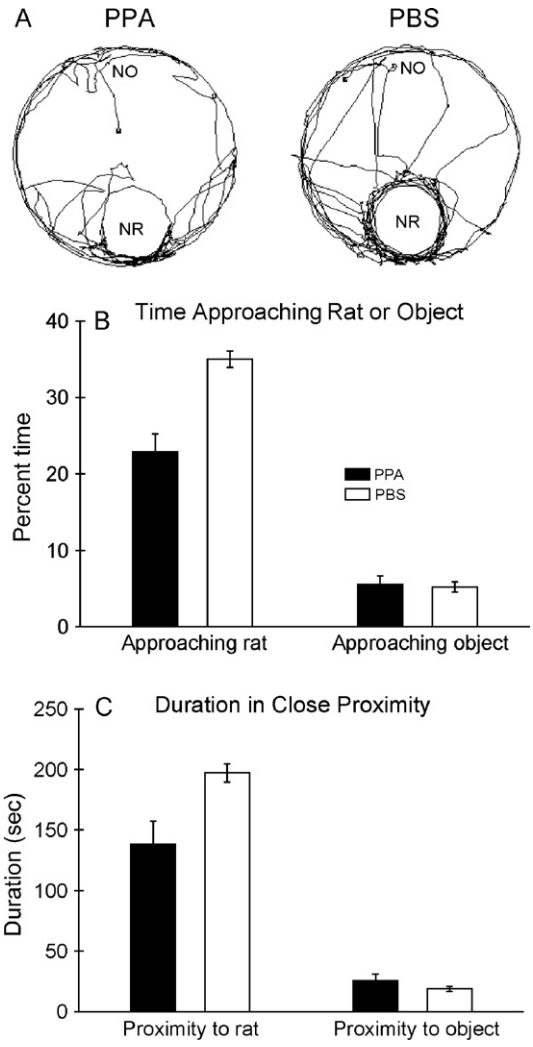

Fig. 2. Novel rat vs. novel object-directed behavior. (A) Representative behavioral movement tracks of PPA and PBS adolescent rats. The tracks were generated by *EthoVision* and represent the rat in each group whose mean distance from the novel rat was closest to the mean value of its group. In each plot the novel object is near the top (designated by NO) and the small cage containing the novel rat is near the bottom (designated by NR). The tracks indicate more locomotion near the caged novel rat by the PBS rat than by the PPA rat. (B) Percent of time spent approaching the novel rat or object (time approaching/total test session time; group mean ± SEM). (C) Duration of time (s) spent in close proximity (within 18 cm) of the novel rat or object. For further details see Section 3.

3.5. Immunohistochemistry

Qualitative image analysis of PPA-treated brains revealed immunohistochemical evidence of reactive astrocytes (GFAP) and activated microglia (CD68) in all regions examined (see Fig. 4). Based on previous findings of increased GFAP and CD68 immunore-

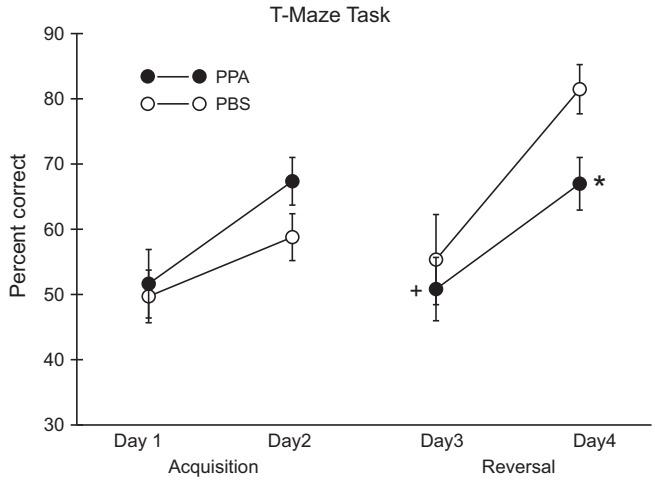

Fig. 3. Group mean (±SEM) percent correct turns in the T-maze task during acquisition (Days 1 and 2) and reversal (Days 3 and 4). + = significantly different from Day 2 performance; * = significantly different from PBS control group. For further details see Section 3.

activity in PPA-treated rat hippocampus and white matter in adult rats [35,36,51,52], we expected increases in GFAP and CD68 immunoreactivity in adolescent rats. ANOVA of data from quantitative image analysis of brain tissue revealed greater GFAP immunoreactivity in the CA1/CA2 region of hippocampus and in external capsule white matter in PPA-treated adolescents than in PBS controls (treatment: $F(1,37) = 3.4$, $p = .028$; Fig. 5A). Analysis also revealed a nonsignificant trend for an increase in CD68 immunoreactive cell counts in these same brain areas of PPA-treated adolescents (treatment: $F(1,37) = 2.5$, $p = .06$; Fig. 5B). There was no qualitative evidence of loss of hippocampal pyramidal cells.

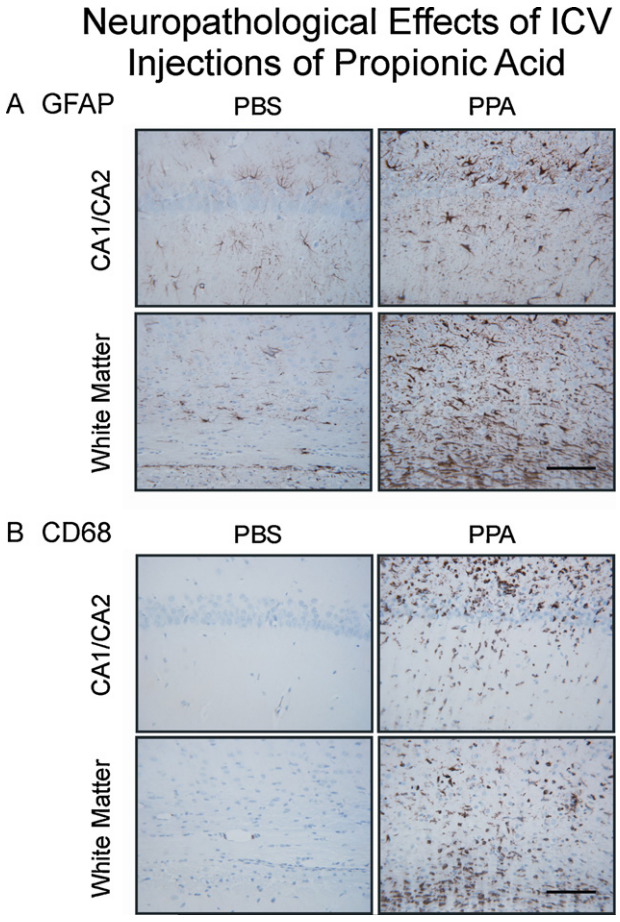

Fig. 4. Representative immunohistochemical photomicrographs of (A) GFAP for astroglia and (B) CD68 for activated microglia of CA1/CA2 hippocampal and external capsule white matter regions of representative adolescent rat brain following intraventricular injections of either PBS vehicle or PPA (scale bar represents 100 μm).

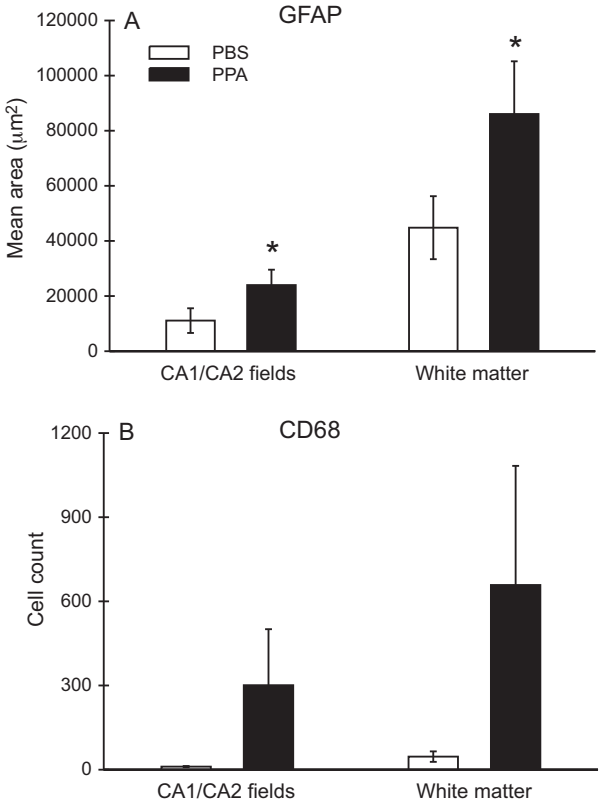

Fig. 5. Group means (±SEM) of immunohistochemical quantification of total GFAP immunoreactivity (A) and CD68 cell counts (B) in hippocampal areas CA1/CA2 and adjacent external capsule white matter. PPA produced significant increases in GFAP immunoreactivity, and nonsignificant trends for increases in CD68 immunoreactivity. * = different from PBS controls ($p < .05$). For statistical details see Section 3.

4. Discussion

The results show that PPA treatment increased restrictive/repetitive behaviors in an object choice test, impaired social behavior in a rat vs. object choice test, and impaired reversal learning in a T-maze task in adolescent rats relative to PBS controls. PPA significantly increased GFAP immunoreactivity in hippocampal areas CA1/CA2 and in white matter adjacent to hippocampus, and there was a nonsignificant trend for similar increases in CD68 immunoreactivity in the same brain structures. These findings reveal additional behaviors in PPA-treated adolescent rats that are similar to our earlier findings with PPA in adult rats [35,51,52] and are consistent with expectations in a rat model of ASD [20,22].

The adolescent period in rats is characterized by a variety of behavioral changes including enhanced social interaction, increased risk taking and novelty seeking, and enhanced reactions to primary rewards, but decreased reactions to aversive properties of stimuli [23,55]. This period has also been characterized as a window of vulnerability to psychopathology [2]. Thus, examination of the effects of PPA is of particular relevance in adolescent rats given the increased levels of social activity at this age relative to younger or older animals [60,61], as well as the greater susceptibility to adverse effects of drugs or toxins on brain reorganization at this age [1,3]. Examination of the effects of PPA in adolescent rats

also broadens the developmental features of PPA-related behavioral effects.

4.1. Restrictive/repetitive interests and behaviors

Restrictive/repetitive interests and behaviors are a core symptom domain and a main diagnostic criterion for ASD. Persons with ASD often exhibit restrictive/repetitive interests and behaviors preferentially toward objects rather than toward social interactions [5,45]. Therefore, restrictive/repetitive interests and behaviors are considered to be important in rodent models of ASD [20,22]. In an earlier study designed to monitor behavior and record brain EEG events after administration of PPA to adult rats, we found that PPA caused abnormal repetitive and stereotyped movements in rats tested in an empty chamber [35]. The current study extended these observations by studying restrictive/repetitive interests and behaviors in adolescent rats in response to a group of novel stimuli objects in an open field test arena. The results showed that PPA-treated adolescents approached, remained closer to, and directed significantly more sniffing behavior toward one particular novel object than the other two novel objects in the arena, relative to PBS controls. These novel findings indicate that PPA can increase restrictive/repetitive interests and behaviors in a test with multiple objects.

4.2. Social behavior

Impairments in social behavior are another core symptom domain in ASD, and are important in animal models of ASD [20,22]. We previously found impairments in social behavior in PPA-treated adult rats tested as same-treatment pairs [51]. The current study extended this finding by studying adolescent rats in a novel rat vs. novel object choice test. Here, PPA-treated adolescents directed significantly less behavior toward the novel rat/more behavior toward the novel object, relative to PBS controls.

Analysis of total distance moved during the social behavior test failed to reveal a difference in locomotor activity between PPA- and PBS-treated adolescents. The absence of a group difference in locomotor activity is consistent with our previous studies showing no effect of PPA on locomotor activity or swim speed during behavioral tests of social behavior or cognition [51,52], suggesting that the effects of PPA on behaviors reported here were unlikely to be confounded by effects of PPA on locomotor activity.

4.3. Cognitive impairment

Failure to reverse a previously learned pattern of rewarded behavior when the reward contingency is reversed is a useful example of restrictive/repetitive behavior, and can be studied using the T-maze or water maze tasks [13,20,38]. Both tasks can be used with a reversal component in which a previously learned response must be suppressed and replaced by a different directional response [38]. For reversal learning both tasks require cognitive mechanisms to recognize that the original reward contingency has been replaced by a new contingency that requires a different directional response. As the water maze is highly stressful and young rats can have difficulty negotiating a large water maze pool [9], here we used a T-maze. As was the case with PPA-treated adult rats tested in the water maze [52], PPA-treated adolescent rats in the present study were not impaired during the acquisition phase, but were impaired during the reversal phase. The absence of impairment during acquisition suggests that cognitive mechanisms for the processing of stimulus and other information required in the tasks were not impaired by PPA. Reversal learning was impaired in the PPA group, which performed at chance during the first reversal session on Day 3, and although they improved by the end of reversal training on Day 4, PPA adolescents remained impaired relative to PBS controls. This suggests that PPA may have impaired a specific cognitive ability underlying learning to suppress a previously learned directional response and the acquisition of a new response.

The occurrence of convulsions in a small number of PPA-treated subjects during T-maze testing was expected, based on our previous finding of epileptiform seizures in a subset of adult rats receiving repeated injections of PPA [35]. In this context it is interesting that seizure disorder is present in approximately 25% of ASD patients [14]. The three PPA adolescents that displayed convulsive behavior were excluded from further T-maze testing and their data were excluded from the T-maze data and histological analyses. This exclusion means that the T-maze results were obtained from rats selected for resistance to PPA-induced convulsions.

4.4. Immunohistochemistry

PPA-treated adolescents showed significantly increased GFAP immunoreactivity in both hippocampal areas CA1/CA2 and in adjacent white matter, and nonsignificant trends for an increase in CD68 immunoreactivity in the same brain areas. These parallel results are consistent with previous findings from our laboratory in adult rats treated with PPA [35,36,51,52], and extend these effects to adolescent rats. Astrocytes are known to be the main CNS cells that metabolize PPA [39], and the findings are consistent with an innate neuroinflammatory response. There was no evidence of gross neuronal loss in hippocampal pyramidal cells, suggesting that PPA is not grossly cytotoxic in hippocampus. These findings are consistent with our previous study, which found no evidence of pyramidal cell loss as measured by direct cell counts and cleaved Caspase 3 immunoreactivity, a marker for apoptosis [35]. This finding is in direct contrast to observations in a number of other neuropathological conditions that produce neuroinflammation together with neuronal loss, such as Parkinson and Alzheimer disease, epilepsy, and AIDS dementia complex [8,46,49]. However, we cannot rule out neurotoxicity effects of PPA in brain areas and cell populations that were not directly examined here.

The neuropathological findings seen in PPA-treated adults [35,51,52] and in PPA-treated adolescents in the current study are consistent with findings from brain tissue of ASD patients. These include reactive astrocytes and activated microglia in hippocampus and neocortex, and changes in white matter, together with little or no change in neuronal cytoarchitecture [59]. Reactive astrocytes and activated microglia release cytokines, including tumor necrosis factor and macrophage chemoattractant protein, which contribute to the neuroinflammatory response and are elevated in ASD [59]. Microglia produce inducible nitric oxide synthetase, leading to the production of nitric oxide, reactive oxygen species, and increased oxidative stress found in widespread brain areas following PPA infusion and in brain homogenates and bloods from ASD patients [18,24,36]. This neuroinflammatory process is particularly localized near the cerebral endovasculature, suggesting that altered permeability of the blood–brain barrier and impaired cerebral blood flow might be components of ASD [56,66]. Specific G-protein coupled receptors, such as GPR41 and GPR43 for short chain fatty acids, have been identified on a number of immune cells including neutrophils, suggesting that PPA may be involved in the activation of the immune response [34].

4.5. Neurophysiological and neuropathological effects of PPA

PPA rapidly altered behavior beginning on the first day of testing in the object-directed behavior task, and continuing throughout behavioral testing on subsequent days. This rapid effect of PPA is consistent with our previous behavioral and electrophysiological findings [35,36,51,52], and is suggestive of rapid physiologi-

cal/biochemical effects that can alter neural function. PPA can inhibit Na^+/K^+ ATPase, increase NMDA receptor sensitivity, promote intracellular calcium release, and elevate nitric oxide, all of which can affect synaptic transmission or neuronal activity [11,21,62,65]. There is evidence linking PPA and related enteric short chain fatty acids with neuroactive effects including effects on neurotransmitter synthesis and release, intracellular pH and calcium homeostasis, gap junctional gating, mitochondrial function, and modulation of gene expression (see [35,36] for reviews). Like PPA, other pharmacological inhibitors of gap junctional gating have been found to produce behavioral effects reminiscent of neuropsychiatric and mood disorders [37,31].

PPA is a weak organic acid that can cross the lipid bilayer of neuronal membranes and cause mild, reversible intracellular acidification, which can produce widespread effects on neurotransmitter release involving glutamate, dopamine, norepinephrine, and serotonin, each of which can influence locomotion and other behaviors [15,47]. Our previous work found that only PPA but not L-propanol, the non-acidic analog of PPA, elicited significant behavioral and electrophysiological effects, suggesting that some pH- or monocarboxylate-dependant mechanism may be important for effects of PPA on behavior or cognition.

In addition, PPA is thought to affect mitochondrial fatty acid metabolism by binding to propionyl coenzyme A and by sequestering carnitine [10,44,62]. Autism has been suggested to be a mitochondrial disorder of impaired fatty acid metabolism [30,25]. Our laboratory has shown that intraventricular administration of PPA or the related enteric short chain fatty acid butyrate produces CNS phospholipid and acylcarnitine profiles similar to those found in bloods of autistic patients [58,64].

PPA, like other short chain fatty acids, is known to alter gene expression [41]. Previously we quantified cyclic AMP responsive element binding protein (CREB) and its phosphorylated and activated form, pCREB, in PPA-treated rat brain and found a significant increase in pCREB immunoreactivity in hippocampus and adjacent white matter [35]. CREB and pCREB were chosen as markers because they are expressed in all CNS cells, they play a role triggering alterations in gene expression by neuronal membrane events, and they are thought to be important in learning and memory mechanisms (see [16] for review). Although not measured here, alterations in pCREB might underlie some of the rapid perseverative behavioral effects found in the present study. These findings are interesting as short chain fatty acids and their derivatives (i.e. valproic acid, an autism associated risk factor), are histone deacetylase inhibitors, providing a plausible mechanism for epigenetic changes found in ASD [41,27].

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