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Intracerebroventricular injection of propionic acid, an enteric bacterial metabolic end-product, impairs social behavior in the rat: Implications for an animal model of autism

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Abstract

Environmental, dietary, and gastrointestinal factors may contribute to autism spectrum disorders (ASD). Propionic acid (PPA) is a short chain fatty acid, a metabolic end-product of enteric bacteria in the gut, and a common food preservative. Recent evidence indicates that PPA can cause behavioral abnormalities and a neuroinflammatory response in rats. Social behavior was examined in similarly-treated pairs of adult male Long-Evans rats placed in an open field following intracerebroventricular (ICV) injection of PPA (4 μl of 0.26 M solution) or control compounds. Behavior was analyzed using both the EthoVision behavior tracking system and by blind scoring of videotapes of social behaviors. Compared to controls, rats treated with PPA displayed social behavior impairments as indicated by significantly greater mean distance apart, reduced time spent in close proximity, reduced playful interaction, and altered responses to playful initiations. Treatment with another short chain fatty acid, sodium acetate, produced similar impairments, but treatment with the alcohol analog of PPA, 1-propanol, did not produce impairments. Immunohistochemical analysis of brain tissue taken from rats treated with PPA revealed reactive astrogliosis, indicating a neuroinflammatory response. These findings suggest that PPA can change both brain and behavior in the laboratory rat in a manner that is consistent with symptoms of human ASD.

Keywords: Intracerebroventricular; Behavioral tracking; Play behavior; Autism; Animal model; Neuroinflammatory; Sodium acetate; Propanol; Short chain fatty acids

1. Introduction

Autism spectrum disorder (ASD) is a class of neurodevelopmental disorders affecting approximately 1 in 166 children (DiCicco-Bloom et al., 2006). Behavioral symptoms associated with ASD include abnormal motor behaviors, repetitive interests and behaviors, cognitive deficits, seizures, and impaired social interactions (Arndt et al., 2005; DiCicco-Bloom et al., 2006). While much research supports a strong multigenetic basis to ASD (DiCicco-Bloom et al., 2006), other evidence suggests that environmental, dietary, and gastrointestinal factors may also contribute to the disorder (Arndt et al., 2005; Horvath et al., 1999; Jyonouchi et al., 2002). There is evidence suggesting that exposure to propionic acid (PPA), 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 bacteria found in the gut

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(Nyhan et al., 1999; Thompson et al., 1990), may be associated with ASD. Anecdotal evidence from parents of ASD children suggests that ASD symptoms increase when their children ingest foods such as refined wheat and dairy products that contain high levels of PPA as a food preservative (Horvath et al., 1999; Jyonouchi et al., 2002). Research has also found that ASD patients may have elevated levels of Clostridia, an early gut colonizer that produces PPA and other short chain fatty acids (Song et al., 2004). Rat models of propionic acidemia or Huntington's disease that make use of PPA or 3-nitropropionic acid (3NP), a derivative of PPA, have yielded brain markers or behavioral symptoms that resemble certain markers and symptoms of human ASD (Borlongan et al., 1995; Brusque et al., 1999; Shear et al., 2000). These include neuroinflammation, neurodevelopmental delay with cognitive deficits, and impaired motor function (Andres, 2002; Vargas et al., 2004; Zwaigenbaum et al., 2005).

Although most of PPA originally accumulates in the gut, PPA readily crosses the gut–blood and blood–brain barriers and can gain access to the CNS. Here it can cross cell membranes and accumulate within cells, inducing intracellular acidification (Bonnet et al., 2000; Karuri et al., 1993), which can alter neurotransmitter release and inhibit gap junctions, potentially altering neuronal communication and behavior (Cannizzaro et al., 2003; Severson et al., 2003). We recently proposed that alterations of PPA levels 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 (MacFabe et al., 2007). We found that ICV injections of PPA induced repetitive behaviors, hyperactivity, turning behavior, retropulsion, kindled seizures, widespread oxidative stress, and a neuroinflammatory response (MacFabe et al., 2008), all of which appear consistent with symptoms of ASD (Andres, 2002; Vargas et al., 2004; Zwaigenbaum et al., 2005). To date no study has investigated the effects of PPA on social behavior. Impairments in social behavior, including impairments in play behavior and other forms of social contact, are among the most salient symptoms of ASD (Arndt et al., 2005; DiCicco-Bloom et al., 2006; Zwaigenbaum et al., 2005). Therefore, we examined the effects of PPA on social behavior in the rat using the same route of administration and dose of PPA employed previously (MacFabe et al., 2007).

2. Methods

2.1. Subjects

A total of 114 adult male Long-Evans hooded rats obtained from Charles River Laboratories (Quebec, Canada) were used. Prior to surgery for implantation of an indwelling guide cannula for ICV injections, 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 °C), and were naïve 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*. Procedures 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. Treatment groups

Following recovery, pairs of rats were randomly assigned to one of four treatment groups, with both rats of each pair receiving the same treatment. The treatment groups were: PPA (4 μl of 0.26 M solution, $n = 30$, 15 pairs); Sodium acetate (SA; 4 μl of 0.26 M solution, $n = 28$, 14 pairs); 1-propanol (PROP; 4 μl of 0.26 M solution, $n = 28$, 14 pairs); and Phosphate buffered saline (PBS; 4 μl of 0.1 M solution, $n = 28$, 14 pairs). 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 our previous dose-response findings (MacFabe et al., 2007). Solutions were buffered to physiological pH 7.5 before injection using hydrochloric acid or sodium hydroxide. Same-treatment pairs of rats were utilized to avoid the potential concern that the control animal in a dissimilar-treatment pair might pursue the treated animal to engage in social interaction while, if the treatment caused a social impairment or social avoidance, the treated animal might flee or otherwise avoid contact with the control animal. Although some studies of social interaction behavior have studied dissimilar-treatment pairs, other studies have successfully studied same-treatment pairs (Slot et al., 2005; Presti-Torres et al., 2007), and a study that included comparison of data from both dissimilar- and same-treatment rat pairs found no significant difference between the 2 experimental designs on measures of social behavior (Pletnikov et al., 1999). Therefore, based on these considerations, a same-treatment design was used here.

2.3. 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 (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. 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).

2.4. ICV injections

Prior to behavioral testing 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 one minute. 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.

2.5. Behavioral test apparatus

Social behavior was evaluated in a circular open-field arena (90 cm diameter, 40 cm high) with Beta Chip bedding covering the floor of the arena. A CD camera and a darkroom lamp were mounted above the centre 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 onto VHS cassettes for later analysis.

2.6. Experimental procedure

On the day before testing, the dorsal surface of one rat from each pair was colored black using black hair dye so that the *EthoVision Tracking System* could distinguish and track each rat separately (Lazar et al., 2008). Rats received a total of two ICV injections of PPA and were injected and behaviorally tested as same-treatment pairs. Within approximately 3–4 min after the first injection, two rats that had been given the same treatment were placed in the open field and behavioral data were collected for 30 min. Following this session, rats were housed individually for approximately one week before again being injected and behaviorally tested in the same way during the opposite phase of the light-dark cycle. The periods of individual housing after surgery and between tests were used because social isolation has been shown to enhance motivation for social interaction (Ikemoto and Panksepp, 1992). Behavioral testing was counterbalanced such that half of the rat pairs in each group were tested first in the light phase and second in the dark phase of the cycle, and the remaining rat pairs were tested in the opposite order. Rats were tested during both phases of the cycle because the rat is a nocturnally active species, and because previous research from our laboratory using a rat model of schizophrenia has found important behavioral differences between the light and dark phases (Lazar et al., 2008). The same rat pairs were used for both behavioral testing sessions.

2.7. Behavioral analyses

EthoVision automatically collected and calculated the following variables of interest for each 10 min of test time:

1. Mean distance apart – mean distance (cm) between the rats in each pair.
2. Proximity – percent of time spent by each pair of rats within 5 cm proximity of each other.
3. Total distance traveled – distance traveled (cm) by each individual rat.

In addition, an experimenter who was blind to the rats' group membership coded the first 10 min of the videotaped records for play behavior using the behavioral measures reported in the rat play behavior studies of Field et al. (2006) and Reinhart et al. (2004). Data for the first 10 min of each test session were coded and analyzed for consistency with the previous methods, and because metabolic clearance of both PPA and SA requires less than 30 min. Each play behavior measure was determined for each similarly-treated rat pair. The play behavior measures were:

1. Frequency of playful initiations – the number of snout to nape contacts.
2. Probability of defense – the number of defenses elicited (withdrawal of the nape from the partners snout) divided by the total number of playful initiations by rat pairs ×100. This measure was obtained only if at least one playful initiation occurred in a rat pair.
3. Type of defense: (a) Probability of evasive defense – the number of evasive defenses, defined as withdrawal of the nape from the partners snout by either leaping, running, or turning away from the partner, divided by the total number of defenses by rat pairs ×100; (b) Probability of facing defense – the number of facing defenses, defined as withdrawal of the nape from the partners snout by turning to face the partner, divided by the total number of defenses by rat pairs ×100. These measures were obtained only if a rat pair displayed at least one defensive response.

2.8. Brain tissue preparation and histological procedures

On the day following the second 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 °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.

In a previous study (MacFabe et al., 2007) our laboratory found that 26 ICV injections of PPA induced neuroinflammatory changes that appeared to be reminiscent of findings in ASD and other neurological conditions such as Parkinsons and Alzheimers disease (Vargas et al., 2004; Whitton, 2007; Zilka et al., 2006). Therefore, to examine whether the greatly reduced PPA injection schedule used in the current study also produced neuroinflammatory changes, 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 analysis.

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, a structure with well-known cytoarchitectonics, and in white matter. Anti-glial fibrillary acidic protein (GFAP; 1:500, rabbit polyclonal, DakoCytomation, Glostrup, Denmark) was used as a marker for reactive astrogliosis. Tissue sections were mounted on glass slides (SurgiPath, Canada) and dried overnight at 37 °C. Sections were deparaffinized and rehydrated using standard immunohistochemical procedures for antigen recovery (Shi et al., 2001). 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 biotinylated antirabbit (Vector Laboratories, Burlingame CA – BA1000) as a secondary antibody 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, 8 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) as well as the *stratum oriens* to *stratum radiatum* were captured at 250× magnification. From the same section of tissue, an additional 7 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 5 randomly selected animals in each group (PPA, $n = 5$; SA, $n = 4$; PROP, $n = 5$; PBS, $n = 5$). Photos 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 staining, the 'area stained' function within ImagePro Plus software was used to quantify 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. Data from images were summed on a per-region basis to yield totals for both the hippocampus (8 images summed per rat) and white matter (7 images summed per rat; Ossenkopp and Mazmanian, 1985).

2.11. Statistical analyses

Mean distance apart, proximity, and total distance traveled data were graphed in 10 min time blocks to reveal behavioral changes across time, and were analyzed with SPSS 13.0 using mixed design analysis of variance (ANOVA) with treatment as the between-subjects factor and time as the within-subjects factor. Simple effects *F*-tests were carried out when appropriate. One-way ANOVA, with treatment as the between-subjects factor, was used to analyze the play behavior measures. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate. Data were analyzed separately for the light and dark phases as they constitute different environmental conditions. Multivariate ANOVA, with treatment as the between-subjects factor, was used for immunohistochemistry analysis. Fisher's LSD post hoc pair-wise comparisons were carried out when appropriate.

3. Results

3.1. Behavior

3.1.1. Mean distance apart, light phase

During the light phase the PPA and SA groups exhibited greater mean distances apart than the PBS control group during most time periods (see Fig. 1A). The PPA and SA groups exhibited comparable mean distances apart during the first 10 min of testing, but by the last 10 min the SA group appeared to be similar to PBS controls, whereas the PPA group exhibited a greater mean distance apart than PBS controls.

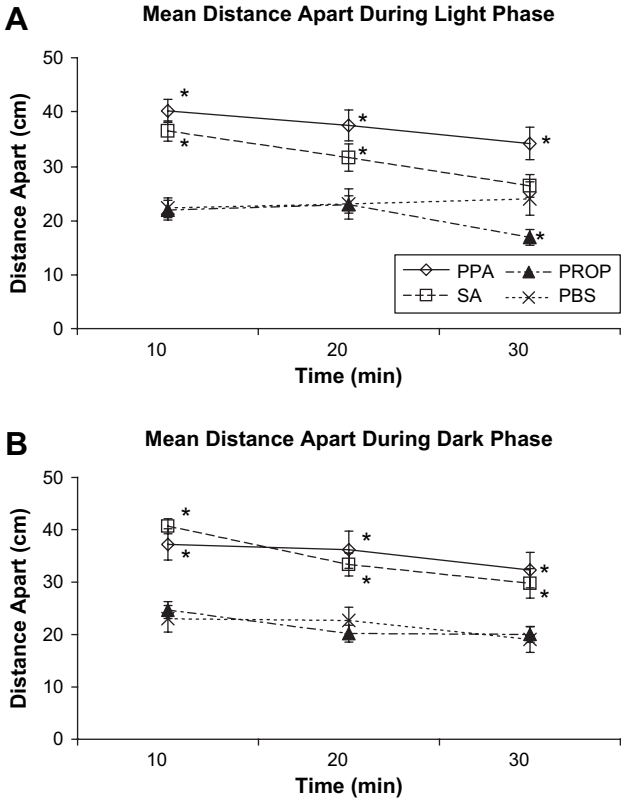

Fig. 1. (A) Mean distance apart (cm) during light phase open-field social behavior testing. (B) Mean distance apart (cm) during dark phase open-field social behavior testing. Data points represent group means of data collected during 10 min periods, and error bars represent ±SEM. * = different from PBS control group at $p < 0.05$ or better. For additional statistical details see Section 3.

These impressions were confirmed by ANOVA, with the finding of a significant time × treatment interaction ($F(6,106) = 4.2$, $p < 0.01$). At the 10 and 20 min intervals, PPA- and SA-treated pairs were significantly further apart than the PBS and PROP pairs ($p < 0.001$). At 20 min, PPA pairs were significantly further apart than SA pairs ($p < 0.01$), and at 30 min, PPA pairs remained further apart than all other groups ($p < 0.001$), while SA and PBS pairs were further apart than PROP pairs ($p < 0.01$). Significant main effects were also obtained for both time ($F(2,106) = 15.1$, $p < 0.001$) and treatment ($F(3,53) = 13.8$, $p < 0.001$).

3.1.2. Mean distance apart, dark phase

During the dark phase the PPA and SA groups exhibited similar mean distances apart throughout the 30 min test period, and both groups exhibited greater mean distances apart than the PBS and PROP groups (see Fig. 1B). As was seen during the light phase, the SA pairs appeared to decrease their mean distance apart at a faster rate than the PPA group. These impressions were confirmed by ANOVA, with the finding of a significant time × treatment interaction ($F(6,106) = 2.6$, $p < 0.05$). Post hoc tests indicated that the PPA- and SA-treated rat pairs were significantly further apart than PBS- and PROP-treated pairs at all three time intervals ($p < 0.001$). In addition, the mean distance between the SA pairs decreased at a more rapid rate ($10 > 20$, 30 min; $p < 0.001$) than the PPA pairs (10, $20 > 30$ min; $p < 0.05$). Significant main effects for time ($F(2,106) = 24.5$, $p < 0.001$) and treatment ($F(3,53) = 11.1$, $p < 0.001$) were also obtained.

3.1.3. Proximity, light phase

During the light phase the PPA pairs spent less time within 5 cm proximity of each other when compared to all other groups throughout the testing period (see Fig. 2A). The SA pairs also spent less time within 5 cm proximity of each other early in the testing session but than became more similar to PBS controls by the completion of testing.

The ANOVA verified these impressions as a significant time x treatment interaction was found ($F(6,106) = 3.5$, $p < 0.01$). Post hoc tests revealed that PPA-treated pairs spent significantly less time within 5 cm proximity of each other than all other groups at all time intervals ($p < 0.05$). SA pairs spent less time within 5 cm proximity of each other than PBS and PROP pairs at the 10 and 20 min time intervals ($p < 0.05$), while at 30 min the SA- and PBS-treated pairs spent less time within 5 cm proximity of each other than PROP-treated pairs ($p < 0.001$). Significant main effects were also obtained for time ($F(2,106) = 22.7$, $p < 0.001$) and treatment ($F(3,53) = 11.7$, $p < 0.001$).

3.1.4. Proximity, dark phase

During the dark phase the PPA and SA pairs spent less time within 5 cm proximity of each other than the PBS and PROP controls (see Fig. 2B). ANOVA confirmed these impressions, as significant main effects were found for treatment

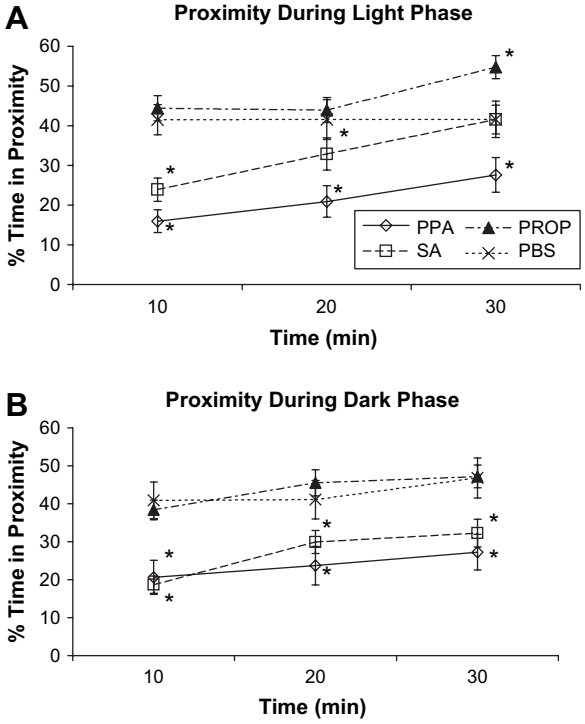

Fig. 2. (A) Percent of time spent by rat pairs within a 5 cm proximity of each other during light phase open-field social behavior testing. (B) Percent of time spent by rat pairs within a 5 cm proximity of each other during dark phase open-field social behavior testing. Data points represent means of data collected during 10 min periods, and error bars represent ±SEM. * = different from PBS control group ($p < 0.05$). For additional statistical details see Section 3.

($F(3,53) = 7.5$, $p < 0.001$) and time ($F(2,106) = 21.8$, $p < 0.001$). Post hoc tests indicated that both PPA- and SA-treated pairs spent significantly less time within 5 cm proximity of each other when compared to the PBS and PROP pairs ($p < 0.05$), and that all pairs within all groups spent more time within 5 cm proximity of each other as testing progressed (30 min > 20 min > 10 min, $p < 0.05$).

3.1.5. Play behavior, light phase

Some rat pairs were not included in all play behavior analyses because of the calculation requirements for the play behavior measures described above. Therefore, in the event that data were not provided by all pairs in a group, the number of pairs that provided data is indicated below in parentheses. During the light phase the PPA and SA groups exhibited fewer playful initiations, were less likely to engage in defense, and were more likely to engage in evasive defenses rather then facing defenses when compared to PBS and PROP controls (see Fig. 3).

ANOVA confirmed these impressions, revealing a significant treatment effect ($F(3,52) = 16.2$, $p < 0.001$) for the measure of *playful initiations*, with PPA- and SA-treated pairs having significantly fewer initiations when compared to the PBS- and PROP-treated pairs ($p < 0.001$; see Fig. 3A). A significant treatment effect ($F(3,51) = 5.7$, $p < 0.01$) was also found for the measure *probability of defense*, with post hoc tests indicating that PPA- ($n = 12$ pairs) and SA-treated rat pairs were less likely to respond to a playful initiation (nape contact) when compared to the PBS ($n = 12$ pairs) and

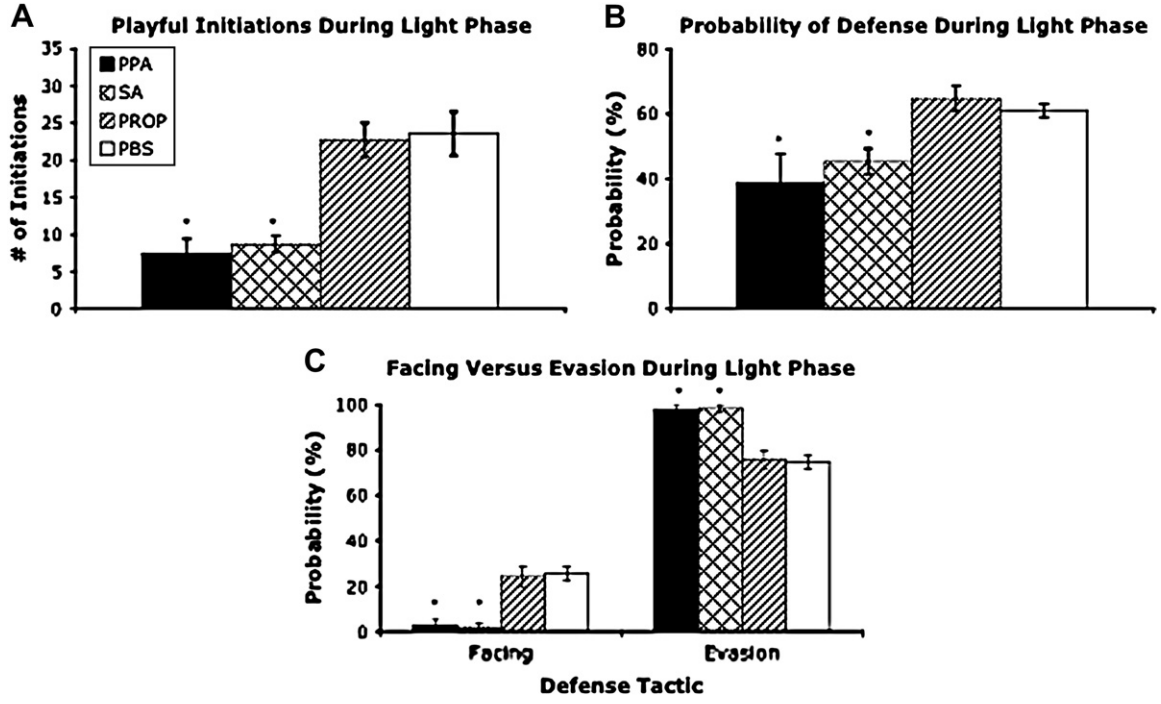

Fig. 3. (A) Number of playful initiations during the initial 10 min of light phase open-field social behavior testing. (B) Probability of defense during the initial 10 min of light phase open-field social behavior testing. (C) Probability of facing versus evasive defense tactics during light phase open-field social behavior testing. Histogram bars represent means of data collected during the first 10 min period of testing, and error bars represent ±SEM. * = different from PBS control group at $p < 0.05$ or better. Refer to legend provided in A. For additional statistical details see Section 3.

PROP pairs ($p < 0.05$; see Fig. 3B). A significant treatment effect ($F(3,48) = 15.5$, $p < 0.001$) was also found for the measure *probability of facing defense versus probability of evasion*, with post hoc tests revealing that the PPA ($n = 9$ pairs) and SA pairs were less likely to engage in facing defenses and more likely to engage in evasive defenses when compared to the PBS ($n = 12$ pairs) and PROP pairs ($p < 0.001$; see Fig. 3C).

3.1.6. Play behavior, dark phase

During the dark phase PPA and SA pairs exhibited fewer playful initiations, SA pairs were likely to engage in defense, and PPA and SA pairs were more likely to engage in evasive defenses rather then facing defenses when compared to PBS and PROP controls (see Fig. 4).

ANOVA confirmed these impressions, revealing a significant treatment effect ($F(3,52) = 23.8$, $p < 0.001$) for the measure of *playful initiations*, with PPA- and SA-treated pairs having significantly fewer initiations when compared to the PBS- and PROP-treated pairs ($p < 0.001$; see Fig. 4A). A significant treatment effect ($F(3,50) = 6.7$, $p < 0.001$) was also found for the measure *probability of defense*, with post hoc tests indicating that SA-treated rat pairs were less likely to respond to a playful initiation when compared to the pairs treated with PBS ($n = 12$ pairs) and PROP ($p < 0.001$); however, PPA-treated pairs ($n = 11$ pairs) did not differ from PBS controls (see Fig. 4B). A significant treatment effect ($F(3,49) = 14.938$, $p < 0.001$) was also found for the measure *probability of facing defense versus probability of evasion*, with post hoc tests revealing that rat pairs treated with PPA ($n = 11$ pairs) and SA ($n = 13$ pairs) were less likely to engage in facing defenses and more likely to engage in evasive defenses when compared to the PBS ($n = 12$ pairs) and PROP pairs ($p < 0.001$; see Fig. 4C).

3.1.7. Total distance traveled, light phase

During the light phase the PROP control group traveled a shorter distance compared to the other groups throughout the 30 min test session (see Fig. 5A). In addition, all groups traveled less distance as a function of time. These impressions were confirmed by ANOVA, which revealed a significant treatment effect ($F(3,110) = 3.6$, $p < 0.05$) with post hoc tests indicating that PROP-treated rats traveled significantly less than the other groups ($p < 0.05$). A significant main effect for time ($F(2,220) = 162.8$, $p < 0.001$) was also found, indicating that the groups traveled less distance over time ($10 > 20 > 30$ min, $p < 0.001$).

3.1.8. Total distance traveled, dark phase

During the dark phase the groups traveled less distance as testing progressed, and there did not appear to be overall group differences (see Fig. 5B). These impressions were confirmed by ANOVA, which revealed a significant main effect for time ($F(2,220) = 165.6$, $p < 0.001$) but no main effect of treatment ($p > 0.05$) or time × treatment interaction.

In sum, PPA-treated rat pairs remained further apart, spent less time within 5 cm proximity of each other, initiated fewer playful interactions, produced fewer defenses, and when defenses occurred, produced more evasive defenses and fewer facing defenses than PBS controls.

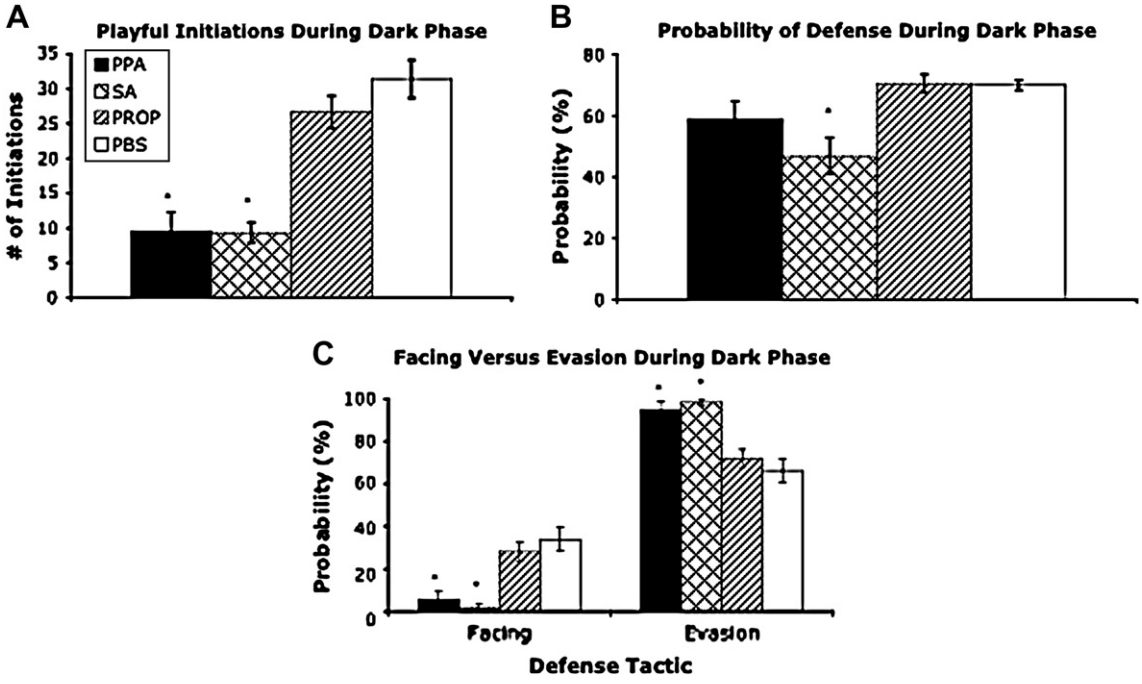

Fig. 4. (A) Number of playful initiations during the initial 10 min of dark phase open-field social behavior testing. (B) Probability of defense during the initial 10 min of dark phase open-field social behavior testing. (C) Probability of facing versus evasive defense tactics during dark phase open-field social behavior testing. Histogram bars represent means of data collected during the first 10 min period of testing, and error bars represent ±SEM. * = different from PBS control group ($p < 0.001$). Refer to legend provided in A. For additional statistical details see Section 3.

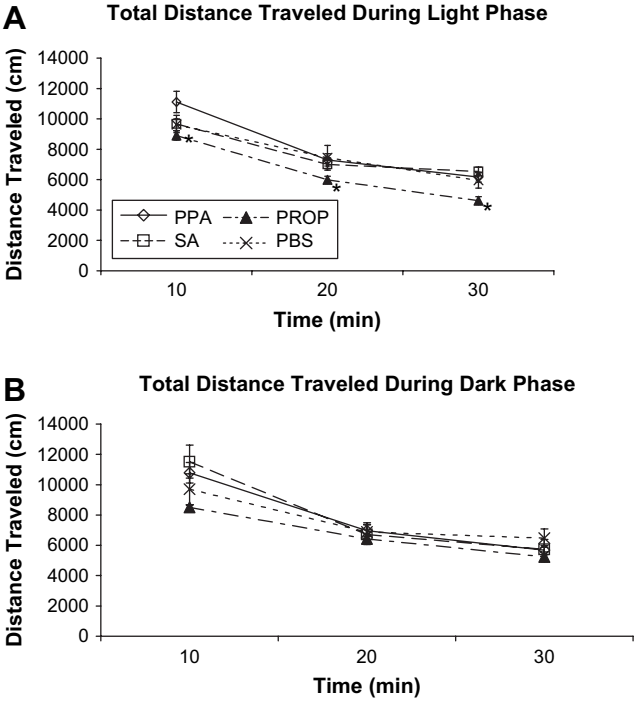

Fig. 5. (A) Total distance traveled during light phase open-field social behavior testing. (B) Total distance traveled during dark phase open-field social behavior testing. Data points represent group means of data collected during 10 min periods, and error bars represent ±SEM. * = different from all other groups ($p < 0.05$). For additional statistical details see Section 3.

3.2. Immunohistochemistry

Qualitative analysis of PPA treated brains revealed hypertrophy of astrocytes in all regions examined, suggesting reactive astrogliosis (see Figs. 6A–D). There were no gross changes in hippocampal pyramidal cells.

Quantitative image analysis of brain tissue revealed a significant effect for treatment in the CA1/CA2 region of the hippocampus ($F(3,15) = 7.618$, $p < 0.01$), with post hoc tests revealing that PPA-treated rats showed significantly increased total mean area of GFAP immunoreactivity compared to all other groups ($p < 0.05$; see Fig. 6E). There was also a non-significant trend for a treatment effect in CA3/DG ($p = 0.094$), with the PPA group showing the greatest total mean area of immunoreactivity.

Counts of the number of GFAP-immunoreactive cells in the CA1/CA2 region for each group were also made from the digital images. ANOVA comparing the counts revealed a significant effect for treatment ($F(3,15) = 3.664$, $p < 0.01$). Post hoc tests revealed that SA-treated rats had significantly fewer ($p < 0.05$) GFAP-immunoreactive cells (mean ± SE = 37.8 ± 2.5) than all other groups, which did not differ (PPA, 54.0 ± 4.24; PBS, 59.2 ± 5.5; PROP, 52.2 ± 4.7).

4. Discussion

The present results indicate that PPA consistently impaired rat social behavior measured as distance apart, proximity, and play behavior. With one exception these outcomes were obtained during both light and dark phases of testing. The only exception was the failure of PPA to significantly alter the probability of defense during the dark phase, relative to PBS controls. However, PPA altered the nature of the defenses that were displayed, with more evasive defenses and fewer facing defenses than PBS controls during both the light and dark phases. In many instances SA produced effects comparable to those produced by PPA. However, the increase in mean distance apart and the reduction in proximity produced by SA were often weaker and of shorter duration than the effects produced by PPA. The shorter duration of effects is consistent with the faster metabolic clearance rate of SA compared to PPA (Brusque et al., 1999). PPA treated rats also showed increased total area of GFAP immunoreactivity in the hippocampal CA1/CA2 region as well as a non-significant trend for an increase of GFAP immunoreactivity in CA3/DG. The fact that PPA did not alter the number of immunoreactive cells in CA1/CA2 suggests that the increase in GFAP-positive total area was likely due to increased GFAP production per astrocyte. Taken together these findings suggest that PPA induced neuroplastic or neuroinflammatory responses characterized by reactive astrogliosis.

Hyperlocomotion may be a confounding factor in studies of social behavior if the hyperlocomotion causes rat pairs to remain at a greater mean distance apart during testing, with consequent reduction in social contact. Although our laboratory previously reported that PPA can cause hyperlocomotion (MacFabe et al., 2007), PPA did not cause hyperlocomotion in the present study, likely due to important procedural differences between the two studies. In our previous study PPA was injected twice daily for seven consecutive days (total, 14 injections), in contrast to the single injections that were given one week apart just before behavioral testing in the present study (total, 2 injections). Also, in our previous study locomotion was measured by placing individual rats in a 40 cm × 40 cm × 30 cm VersaMax Plexiglas open field, whereas in the current study rats were tested as pairs in a larger circular arena (90 cm diameter open field). These methodological differences may account for the occurrence of hyperlocomotion in the MacFabe et al. (2007) study but not in the current study. Whatever the explanation for the different outcomes, the fact that PPA did not cause hyperlocomotion here suggests that the effects of PPA on social behavior were not secondary to effects of PPA on locomotion. The fact that rats treated with PROP displayed normal social behavior together with mild hypoactivity is consistent with past findings from other alcohol treatments (e.g. ethanol; Correa et al., 2003).

PPA and SA induced similar changes in some social behavior measures, while PROP, a non-acidic alcohol homologue of PPA, resulted in few behavioral changes, and those that it did alter were not similar to the changes produced by the carboxylic acids. This suggests that the acidic properties of PPA and SA may have been important for the behavioral changes found in the current study. Support for this idea comes from past studies that have found associations between acidosis and changes in social and other behaviors. For example, acidosis

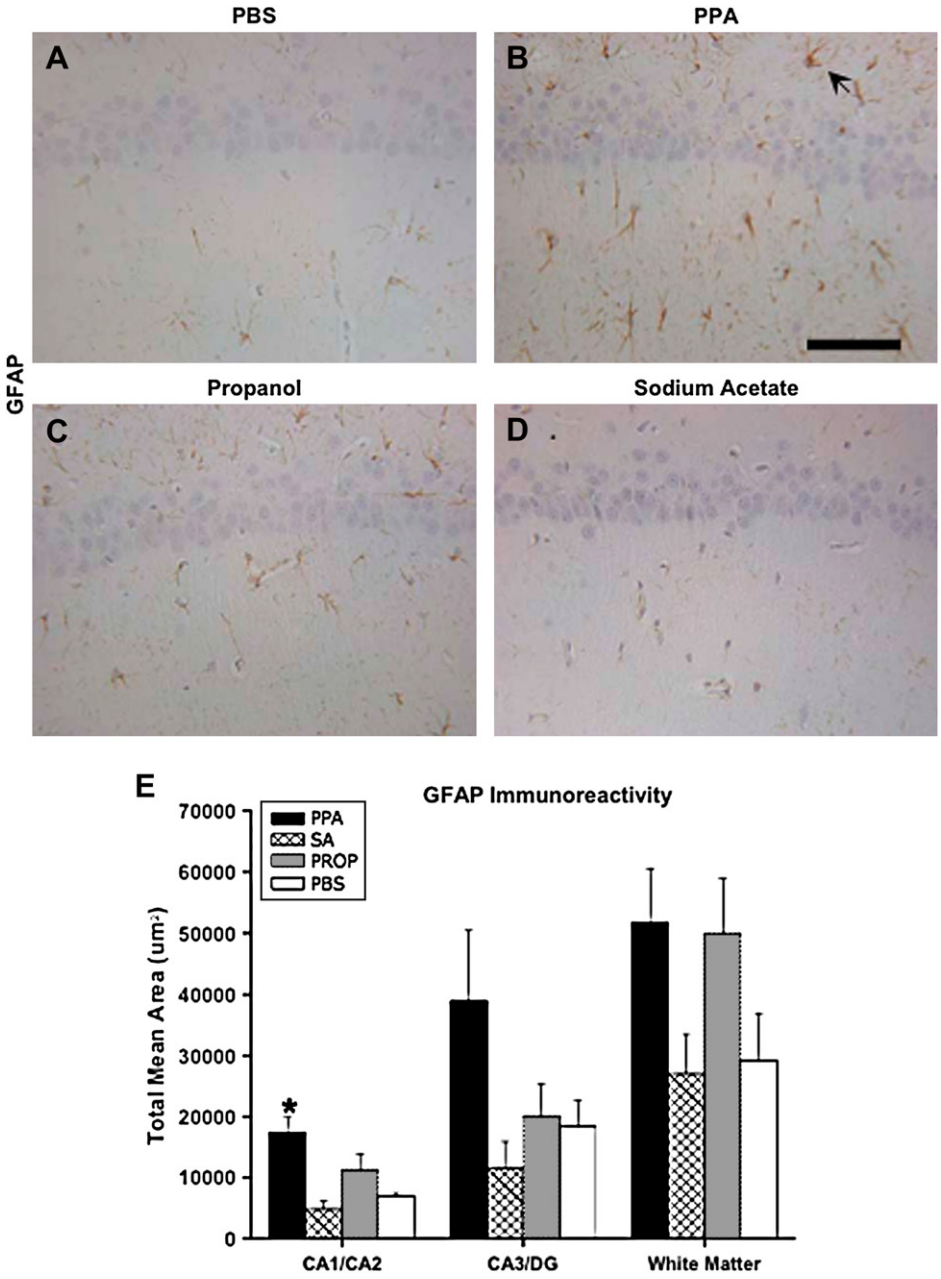

Fig. 6. (A–D) Representative photomicrographs showing glial fibrillary protein (GFAP) immunoreactivity in dorsal hippocampus (CA2 region) from rats that received single ipsilateral intraventricular injections of PPA, PBS, PROP, or SA control solutions once a week for two weeks. Only PPA produced reactive astrogliosis (arrow). (E) Bar graphs representing immunodensity quantification of GFAP immunoreactivity in hippocampal regions (CA1/CA2, CA3/DG) and adjacent external capsule white matter. Only PPA produced significant increases in GFAP immunoreactivity in CA1/CA2 compared to controls. * = different from all other control groups ($p < 0.05$). Original magnification 40×; scale bar represents 100 μm. For additional statistical details see Section 3.

in the rat involving an increase in both PPA and acetate caused by fermentation of carbohydrates in the gut, altered social behavior in a social interaction test (Hanstock et al., 2004). Cases of acidosis in humans often involve symptoms of confusion, agitation, and other altered behaviors (Puwanant et al., 2005).

PPA is capable of altering a number of neurotransmitter systems including dopamine, calcium, and of particular interest to the current findings, serotonin (5-HT; Cannizzaro et al., 2003; Mitsui et al., 2005). Previous research has found that the presence of PPA and other fatty acids increases 5-HT release in the gut (Mitsui et al., 2005). In the brain, PPA and SA can enter brain cells and accumulate intracellularly, where they can indirectly increase 5-HT release by the reduction of intracellular pH (Bonnet et al., 2000; Karuri et al., 1993; Severson et al., 2003). Alterations in 5-HT levels have been implicated in social behavior. For example, a study investigating social interaction in rats discovered that administration of a 5-HT receptor agonist reduced levels of social investigation, and that a 5-HT antagonist reversed these effects (Gonzalez et al., 1996). In addition, Kalueff et al. (2007) discovered

that mice with enhanced 5-HT availability exhibited reduced social behavior. Therefore, it appears that PPA and SA may affect social behavior by increasing 5-HT levels through the process of intracellular acidification.

While PPA and SA appear to posses the ability to alter social behavior through the process of intracellular acidification, intracellular PPA differs from SA in that it has the ability to inhibit carnitine function, which is vital for normal metabolism of fatty acids (Brass and Beyerinck, 1988). Organic acids that collect within the cell create a buildup of intracellular acyl-Coenzyme A (CoA), which interrupts metabolism (Brass and Beyerinck, 1988). Carnitine assists fatty acid metabolism by removing the acyl groups and releasing the CoA (Brass and Beyerinck, 1988). As a result of inhibiting carnitine function, PPA can further decrease cytoplasmic pH via the accumulation of SA and other fatty acids (Bonnet et al., 2000; Brass and Beyerinck, 1988). Therefore, in the presence of PPA, the exposure and resulting accumulation of other short chain fatty acids may contribute to and worsen what are already detrimental symptoms. Consequently, the results of these studies may also be relevant to other disorders associated with increases of PPA and/or other short chain fatty acids.

The finding that PPA induced neuroplastic or neuroinflammatory responses characterized by reactive astrogliosis is similar to, but not as robust as, the GFAP immunoreactivity seen in rats treated with PPA for 7 or 13 consecutive days (MacFabe et al., 2007, 2008). Both of these treatment regimes showed reactive astrogliosis and activated microglia in hippocampus and neocortical white matter, in the absence of apoptotic neuronal loss of pyramidal neurons. However it is important to note that no observable increase in activated microglia, as measured by CD68 or IbA1 staining, occurred in the current study (data not shown). This suggests that increased GFAP immunoreactivity in astrocytes may precede activation of ''resting'' microglia (IbA1) or recruitment of peripheral macrophages and their transformation into activated microglia in the CNS (Mittelbronn et al., 2001; MacFabe et al., 2008). Alternatively, the shorter duration of PPA treatment may have been insufficient to generate such a response.

Similar inflammatory responses have also been found in diseases such as Parkinson's and Alzheimer's with some evidence suggesting that this response may impair brain function (Whitton, 2007; Zilka et al., 2006). Studies investigating the role of the hippocampus and cingulate have found that both appear to be involved to some degree in social function (Becker et al., 1999; Brennan and Kendrick, 2006; Grady and Keightley, 2002). Consequently, PPA may be inducing a neuroinflammatory response that impairs the function of structures involved in social behavior, such as the hippocampus and cingulate. However, as the social effects in the current study are seen immediately following injection, it is unlikely that a neuroinflammatory response would play as prominent a role as a fast-acting mechanism such as 5-HT. Nonetheless, future research is needed to further investigate the extent that these underlying mechanisms contribute to the social deficits induced by PPA.

4.1. Relation to ASD

In past studies, increased distance apart and less time within a defined proximity have been used to support social behavior impairments in animal models (Lazar et al., 2008). For example, in a rat model of schizophrenia Lazar et al. (2008) found that rat pairs exposed to nerve growth factor during early development maintained a greater mean distance apart and spent less time within 20 cm proximity of each other when compared to controls. The details of social interaction in rats also have been studied, with decreases in the measures playful initiations, probability of defensive response, and probability of turning defense all viewed as reductions in play behavior (Field et al., 2006; Reinhart et al., 2004). In addition to animal studies, experiments investigating human disorders, including ASD, have used similar variables to measure social behavior (Nikopoulos and Keenan, 2003). For example, several experiments evaluating treatment effectiveness in children with ASD interpret increases in the number of social initiations as behavioral improvements (Nikopoulos and Keenan, 2003). Therefore, the findings and conclusions of the current study are consistent with past studies examining abnormal social behavior symptoms in animals and human ASD patients.

In addition to the similarities between the social impairments found in the current study and the social abnormalities seen in human ASD, the proposed mechanisms underlying these deficits are also associated with ASD. For example, research examining the role of 5-HT in ASD patients found elevated levels of plasma 5-HT, 5-HT abnormalities affecting early cortical development, and improvements in a number of symptoms following treatment with medications that act on the 5-HT system (Chugani, 2004; McDougle et al., 1996). Further, the neuropathological findings in the PPA rat model (MacFabe et al., 2007) are similar to findings from brain tissue of ASD patients where autopsy results include activated microglia and reactive astrocytes in hippocampus, neocortex, and white matter, coupled with comparatively minor effects in neuronal cytoarchitecture (Bauman and Kemper, 2005; Vargas et al., 2004).

Male rats were used in the current study because ASD is more prevalent in males (Trottier et al., 1999). Some research suggests that females may be less vulnerable to PPA and related compounds as estrogens may be protective in disorders of increased oxidative stress and neuroinflammation (Wilson et al., 2006). For example, administration of 3NP to male rats induced striatal lesions and abnormal motor symptoms, whereas females were resistant to these effects, possibly through protection by estrogen (Nishino et al., 1998). In addition, colonic short chain fatty acids, including PPA, affect serum cholesterol in males but not in females. This suggests that sex differences in systemic lipid metabolism may be related to a more pronounced response to PPA by males (Wolever et al., 1996). Further research is needed to determine whether female rats are less susceptible to PPA in the context of an animal model of ASD.

Other animal models of ASD have been reported. Pletnikov et al. (1999) infected rats with Borna virus and found impaired

play behavior and social interaction using an experimental design similar to that used in the current study. Early exposure to valproic acid is associated with elevated levels of PPA and leads to locomotor and repetitive hyperactivity, decreased social behavior, and diminished prepulse inhibition, all of which are typical of ASD (Schneider and Przewlocki, 2005; Schulpis et al., 2001). This suggests that findings reported in studies using valproic acid could be related to elevated PPA. Regarding molecular mechanisms of social behavior, Lim et al. (2004) showed that manipulation of a single gene to enhance expression of the vasopressin V1a receptor dramatically increased social partner preference in the vole, suggesting a possible mechanism for changes in social behavior.

The results of the present study show that administration of PPA impairs social behavior by reducing inter-animal contact and altering social responses to contact. Our earlier work with this model showed that PPA induces repetitive movements and abnormal motor behaviors (MacFabe et al., 2007), and recent findings indicate the presence of cognitive impairment and repetitive interests in PPA-treated rats tested in a water maze task (Shultz et al., 2007). Taken together, this evidence suggests that PPA may alter preexisting neural circuits in the adult rat to impair complex behavior in a way that is consistent with a rat model of autism.

In summary, direct ICV injections of PPA in adult male rats induced social behavior impairments that were not secondary to hyperlocomotion. As PPA and SA induced similar changes in some social behavior measures, it seems possible that the acidic properties of the compounds may have been responsible for the impairments. The social behavior impairments found in the current study are consistent with symptoms of human ASD and support the use of PPA in an animal model of ASD. Further research 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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