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Behavioural and brain ultrastructural changes following the systemic administration of propionic acid in adolescent male rats. Further development of a rodent model of autism

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Abstract

Short chain fatty acids, produced as gut microbiome metabolites but also present in the diet, exert broad effects in host physiology. Propionic acid (PPA), along with butyrate and acetate, plays a growing role in health, but also in neurological conditions. Increased PPA exposure in humans, animal models and cell lines elicit diverse behavioural and biochemical changes consistent with organic acidurias, mitochondrial disorders and autism spectrum disorders (ASD). ASD is considered a disorder of synaptic dysfunction and cell signalling, but also neuroinflammatory and neurometabolic components. We examined behaviour (Morris water and radial arm mazes) and the ultrastructure of the hippocampus and medial prefrontal cortex (electron microscopy) following a single intraperitoneal (i.p.) injection of PPA (175 mg/kg) in male adolescent rats. PPA treatment showed altered social and locomotor behaviour without changes in learning and memory. Both transient and enduring ultrastructural alterations in synapses, astro- and microglia were detected in the CA1 hippocampal area. Electron microscopic analysis showed the PPA treatment significantly decreased the total number of synaptic vesicles, presynaptic mitochondria and synapses with a symmetric active zone. Thus, brief systemic administration of this dietary and enteric short chain fatty acid produced behavioural and dynamic brain ultrastructural changes, providing further validation of the PPA model of ASD.

KEYWORDS

brain, electron microscopy, propionic acid

1 | INTRODUCTION

Autism spectrum disorders (ASDs) are highly heritable, complex and pervasive conditions, characterized by communication deficits, restricted interests, emotional problems and cognitive impairments. Many genes traditionally thought to be involved in autism encode cell adhesion molecules and proteins involved in neurogenesis, synaptogenesis, synaptic functions and other cellular processes (Chen, Chang, & Huang, 2019; Guang et al., 2018; Zhao et al., 2018). In addition to the genetic component, a number of environmental factors participate in autism pathogenesis, affecting synapse activities, neuroimmune regulation, gastrointestinal function, metabolism and gene expression (Choi et al., 2018; Huang & Jin, 2017; MacFabe, 2015; Rizzetto, Fava, Tuohy, & Selmi, 2018). Therefore, most of such conditions represent the consequence of interaction between individual's genetic profile and the environment

that he/she is exposed to (Blatt, 2012). Emerging research has shown microbiota and their metabolites dynamically affect host metabolic, immune, behavioural processes and gut-brain communications throughout the lifecycle (Bilbo, Block, Bolton, Hanamsagar, & Tran, 2018; Kim, Yun, Oh, & Choi, 2018; MacFabe, 2015; Sharon et al., 2019; Shenderov & Midtvedt, 2014; Slattery, MacFabe, Kahler, & Frye, 2016;). The relationship between gastrointestinal symptoms and many neurological states, such as anxiety, depression, stress and ASD is shown in numerous studies (Foster, Rinaman, & Cryan, 2017; Fung, Olson, & Hsiao, 2017; Ishii et al., 2019). Correspondingly, various animal models of ASD have been proposed by modulating gut microbiome and metabolite level (Kim et al., 2018; MacFabe, 2015).

Short chain fatty acids, which are produced following the dietary fermentation of carbohydrates and some sugars, but also present in diet, have been shown to play broad roles in host cellular physiology in health and disease. Propionic acid (PPA), one of the main short chain fatty acids, plays particular role in the development, metabolism and immunity in health, but also in some inherited and acquired diseases, including those which affect brain function and behaviour (MacFabe, 2015; Nankova, Agarwal, MacFabe, & La Gamma, 2014; Venema, 2017, 2018). As a weak acid, PPA is absorbed passively in the gut, liver and brain, is up taken systematically, both passively and actively (monocarboxlate transporters) and activates G-protein coupled receptors (Conn, Fell, & Steele, 1983; Karuri, Dobrowsky, & Tannock, 1993; MacFabe, 2015). PPA has broad positive effects: it acts as a tumour suppressor, modulates the function of the neuroendocrine system and anti-inflammatory processes, affects CREB, synaptic proteins, lipids, participates in gene regulation and apoptosis (MacFabe et al., 2007; Sivaprakasam, Prasad, & Singh, 2016; Venema, 2017, 2018). However, excessive levels of PPA are known to produce developmental delay, oxidative stress and metabolic or immune disturbances, which have some similarities with propionic acidemias and autism (de la Bâtie et al., 2018; Nankova et al., 2014; Pfeifer, Tassel, & Miller, 2018). High levels of *Clostridia, Desulfovibrio* or *Bacteroidetes* (bacteria, which produce PPA directly or indirectly), frequently present in the gut of ASD patients (Finegold, Summanen, Downes, Corbett, & Komoriya, 2017; Gora et al., 2018). In addition, PPA is often added to processed carbohydrate containing foods many ASD children crave (Sasaki et al., 2016; Scotter, Thorpe, Reynolds, Wilson, & Strutt, 1996), which, in addition to gastric absorption, further increase the additional production of this fatty acid in the gut microbiome (MacFabe, 2015; Scotter et al., 1996).

Further to this we (Lobzhanidze et al., 2019; MacFabe, 2015; MacFabe et al., 2007; Shams, Foley, Kavaliers, MacFabe, & Ossenkopp, 2019; Shultz et al., 2008, 2015), and others (Aabed et al., 2019; Choi et al., 2018; El-Ansary et al., 2018) have used the exposure of PPA and related short chain fatty acid administration in rodents as an acceptable animal model of autism. It was shown that the rats, treated with PPA (intracerebroventricular, intraperitoneal, subcutaneous injections, intragastric gavage), exhibit many autism-like behaviours, like repetitive, antisocial, anxiety-like and repetitive behaviours, including dystonia and seizures.

Highlights

- Propionic acid affects the ultrastructure of the hippocampus in adolescent rats
- Propionic acid affects the structural parameters of hippocampal synapses
- Propionic acid affects the ultrastructural parameters of presynaptic mitochondria

Brains from PPA-treated rodents show micro- and astroglial activation, mitochondrial and fatty acid dysfunction, increased the levels of neurotoxic cytokines and oxidative stress markers, and other alterations, consistent with the findings in human ASD (Choi et al., 2018; El-Ansary et al., 2018; MacFabe et al., 2007; Mepham et al., 2019; Nankova et al., 2014; Shams et al., 2019). In addition to animal models, autism-like biochemical alterations were found on PPA-treated human lymphoblast cell lines (Frye et al., 2016, 2017; Nankova et al., 2014; Rose et al., 2017) and human stem cells (Abdelli, Samsam, & Naser, 2019; Yang et al., 2019, Neurochemistry, accepted for publication).

Although PPA-mediated autism-like neurochemical and behavioural effects have been described in many studies, only a few reports of morphological consequences of PPA on the brain are available (Choi et al., 2018; Lobzhanidze et al., 2019; MacFabe et al., 2007; MacFabe, Cain, Boon, Ossenkopp, & Cain, 2011). Moreover, the description of the brain ultrastructure in the PPA and other animal models of autism are lacking. Therefore, it is not clear how PPA-mediated effects are translated into specific ultrastructural elements of the brain. Traditionally ASD is considered a disorder of synaptic dysfunction and cell signalling with neuroinflammatory and neurometabolic components (Dipasquale et al., 2017; Guang et al., 2018; Pardo & Meffert, 2018; Siniscalco, Schultz, Brigida, & Antonucci, 2018). Therefore, the elucidation of the fine structure of synapses, other forms of cell signalling, mitochondria and possible metabolic alterations in the PPA-treated brain should be of special importance.

In the present research, we utilized behaviour analysis and electron microscopy (EM) in order to evaluate social

behaviour, spatial reference memory, locomotor activity and the ultrastructure of the hippocampus and medial prefrontal cortex in PPA-treated male adolescent rats. Both regions play important role in social and motor behaviour, cognition, anxiety, memory and impulse control (Ariza, Rogers, Hashemi, Noctor, & Martinez-Cerdeno, 2018; Cooper et al., 2017; Cooper & Simons, 2019; Hashemi, Ariza, Rogers, Noctor, & Martinez-Cerdeno, 2017) and are known to be involved in autism pathogenesis. Moreover, in both areas of PPA-treated brain ASD-like light microscopic and biochemical alterations were described (MacFabe et al., 2007). Unlike previous PPA studies, we used Wistar rats versus Long-Evans and single and small dose of systemic PPA versus animal chronic treatment.

2 | MATERIAL AND METHODS

2.1 | Animal housing and treatment

The experiments were performed on adolescent (P30-35) male Wistar rats, weighing 110–115 g. The Animals were housed 3/cage (37 × 30 × 25 cm), in a controlled environment (temperature 20–22°C, humidity—55%–60%, 12-hr light/dark cycle) and had free access to food and water. Just before the treatment, the rats were randomly assigned to experimental ($n = 12$) and control groups ($n = 12$). Experimental rats received single i.p. injection of PPA (Sigma-Aldrich, MO, USA) dissolved in 0.1 M PBS, at a dose 175 mg/kg, pH—7.4 (Choi et al., 2018; MacFabe et al., 2007; Ossenkopp et al., 2012). Control rats received single i.p. injection of the same dose of Phosphate-buffered saline (PBS). The animals were treated between 9:00 and 10.00 a.m. EM study was performed on five rats from each group; the remaining animals were used in other studies. The timetable of experiments is shown in Figure 1.

2.2 | Behavioural analysis

2.2.1 | Assessment of spatial reference memory in Morris Water Maze (MWM)

Spatial reference memory was assessed 60 min after the PPA treatment using the adapted version of "hidden-platform" MWM test (de Quervain, Roozendal, & McGaugh, 1998; Ge et al., 2010; Vorhees & Williams, 2006; Wong et al., 2007). The maze was a circular steel tank, 1.5 m in diameter and 0.5 m in height. It was filled with water (22 ± 1°C), which was made opaque with a non-toxic dye. The room, in which the pool was located, had a sufficient number of spatial cues (door, window, posters on the walls, etc.) for the test animal. The pool was divided into four quadrants by two "imaginary" lines. The end of each line demarcated four cardinal points: North (N), South (S), East (E) and West (W). These points are not true compass directions but refer to S as experimenter's position, N—opposite point, E—the experimenter's right and W—the experimenter's left (Vorhees & Williams, 2006), The escape platform (diameter 4.5 cm) was submerged in the southwest quadrant (SW), 1.3 cm below the water surface at a fixed position (Figure 2).

The test consisted of a 1-day acquisition phase and the retrieval phase. The acquisition phase consisted of two trials; each comprised four releases of the test animal from different

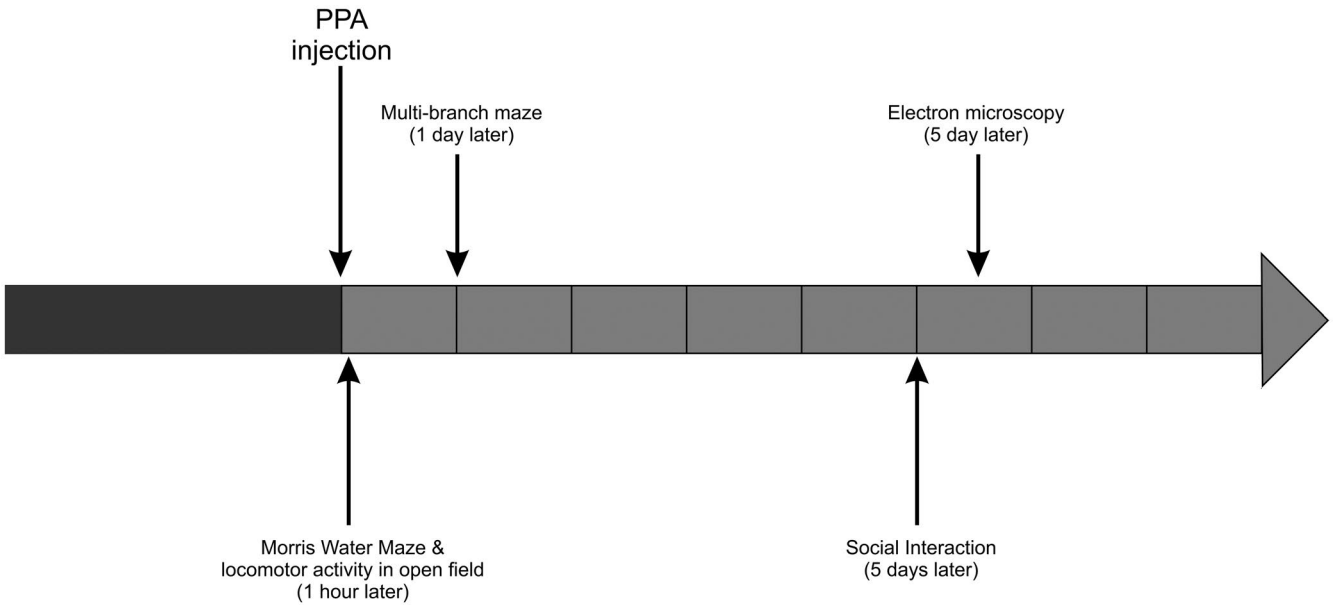

FIGURE 1 The time-table of behavioral and electron microscopic experiments

positions: N, W, S and E (Vorhees & Williams, 2006). The rat was placed into the water, facing the wall from one of the abovementioned positions and was allowed to swim for a maximum of 60 s until the platform was found. When the rat reached the platform, it was required to remain there for 30 s. If the rat did not locate the platform within 60 s, the rat was gently guided to the platform, where it remained for 30 s. After the completion of both trials, the rats were returned to their home cage.

The retrieval phase was performed 30 min and 24 hr after the acquisition phase. The platform was removed. Memory retention was determined using single 60 s trial. The rat was placed into the water, facing the wall from the novel points: A (after 30 min) and B (after 24 hr), opposite to those where the platform was previously located. During 60 s, the rat explored the environment. The behavioural parameters, such as swimming distance, swimming velocity, the time spent in each quadrant, and the latency in the "target" quadrant (where during acquisition phase the platform was located), were monitored and recorded by a digital video camera, placed above the pool. The camera was interfaced to the computer. The data were stored into Excel files and processed with *Minitab 17* statistical software.

2.2.2 | Assessment of learning in Multibranch Maze (MBM)

This learning paradigm was studied 24 hr after treatment in modified radial arm maze—multi-branch maze (MBM). The test was described in our previous study (Bikashvili et al., 2012). Briefly: MBM (total size—45 × 8 × 30.5 cm) consisted of *10 equally spaced arms*, mounted on props of 30 cm height was used. On one corner of the maze, the starting point was located, on the opposite side—the nest-box. Some arms were "closed," the others were "open." One "open" arm led to the nest-box (Figure 3). Each rat performed the test for 10 days. Each day the rat had five trials: 5 min for each trial. At the beginning of each trial, the rat was placed on the starting point and allowed to find the optimal trajectory to the nest-box by trial and error. Each passage of the "open" arm, from which the animal had the opportunity to find the correct direction, served as a stimulus for further movement. Getting in a "closed" branch was considered as an error and forced the rat to search for the correct way. The deliverance from non-aetiological conditions (the maze) or "the hit into a nest-box," was considered as a reward or the motivation for maze learning. The learning was assessed by the test performance within each trial: the time needed to cover the

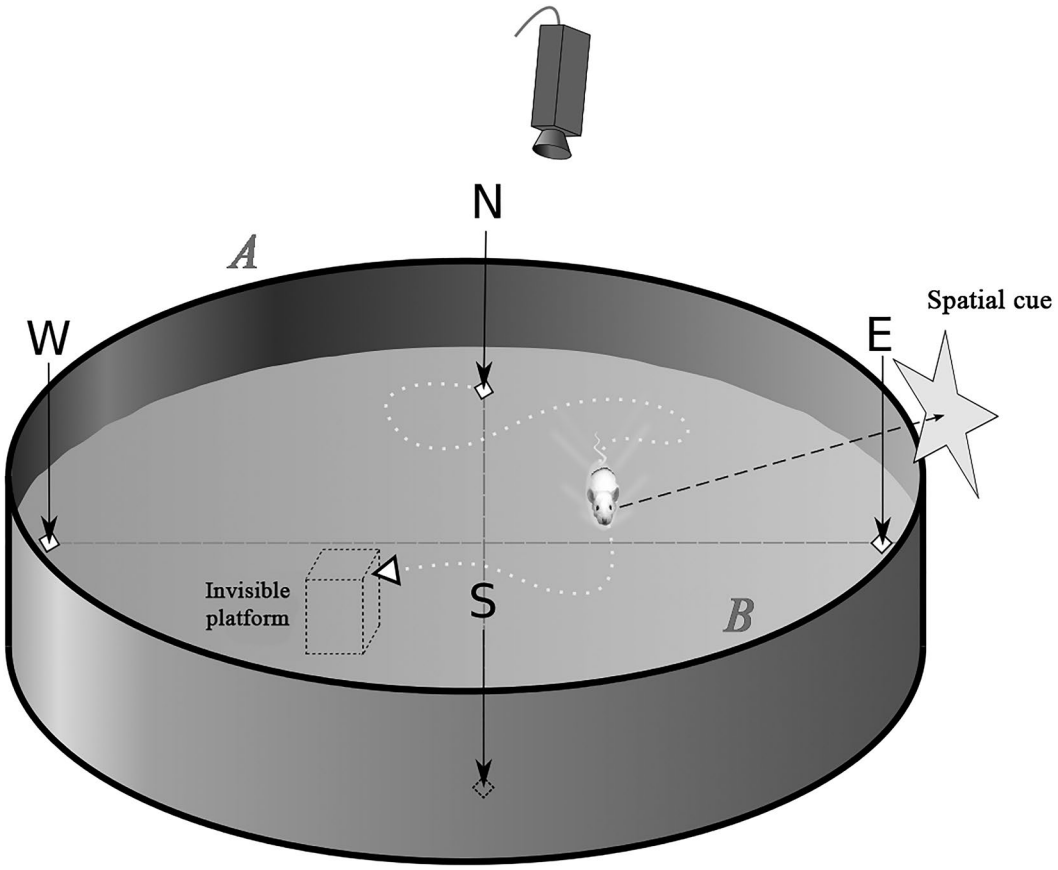

FIGURE 2 Schematic image of MWM, used in the present study

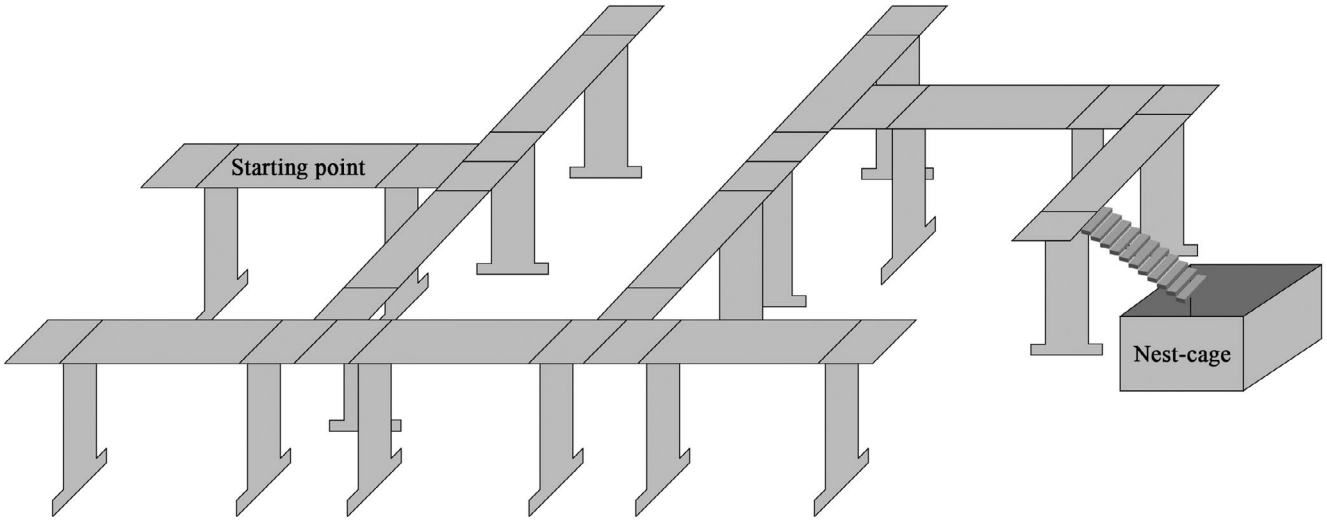

FIGURE 3 Schematic image of MBM, used in the present study

distance between the starting point and nest-cage and the number of "errors" (the visits in "closed" branches) were measured. The results indicate the average (mean ± *SEM*) of all measurements for the entire 10-day period.

2.2.3 | Assessment of locomotor activity in the open field (OF)

The locomotor activity was measured 60 min after treatment, in the square OF (70 × 70 cm). The dark surface of OF was marked with horizontal and vertical white lines with 6 mm thickness. These lines made the squares with 14 × 14 cm dimensions. Each rat performed the test for 10 min. The behaviour was recorded by a video camera attached to the computer. Locomotor activity was evaluated based on how many times the rat crossed white lines.

2.2.4 | Assessment of social interaction

In our earlier study, we described social interaction 120 min after the same regime of treatment (Lobzhanidze et al., 2019). In the present study, we evaluate the effect of PPA 5 days (d) after treatment, using modified three-chamber apparatus (Crawley, 2005; Lee, Park, Lee, & Han, 2018; Nadler et al., 2004). The size of the apparatus was 45 × 10 × 21 cm. In one corner of the apparatus, the wire cage (10 × 10 × 10 cm) for conspecific rat (social stimulus) was placed. The wire cage allowed visual, olfactory, auditory and some tactile contact between the subject and the stranger rat. In another corner, translucent chamber with unfamiliar inanimate object (non-social stimulus) was located. The third chamber was the central area. The session started with placing the PPA or PBS-treated rat in the central chamber for a 10 min habituation. During this period, the apparatus was empty. After 10 min, the stranger rat and unfamiliar inanimate object were placed in their left/right locations and the subject rat was allowed to explore the space during for 10 min and to visit social stimulus or non-social stimulus. The behaviour was video recorded. Social interaction was measured by (i) the number of visits of the subject rat into the chamber with conspecific rat and the number of visits to the inanimate object and (ii) the time spent by the subject rat in sniffing the cage with stranger rat and the time spent with inanimate object. In this way, social motivation defined as the tendency to spend time with a novel rat versus a novel object was evaluated.

2.2.5 | Statistical analysis of behavioural data

Statistical analysis was carried out in a blind manner. Numerical data of tests were calculated by means of statistics package Minitab 17. The two-sample *t* test was used in order to determine the difference between PPA- and PBS-treated rats. The *p*-value of less than .05 was considered statistically significant. The data are presented as a mean ± *SEM*.

2.3 | EM analysis

On the 5th day after PPA injection, the animals were sacrificed and brain samples collected for EM examination. Conventional technique, described in our earlier studies was used (Lobzhanidze et al., 2019). Briefly: after pentobarbital injection (100 mg/kg), five control and

five experimental animals underwent transcardiac perfusion with ice-cold heparinized 0.9% NaCl, followed by 500 ml of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at a perfusion pressure 120 mm Hg. The left hemispheric brain tissue blocks containing the hippocampus and medial prefrontal cortex were cut into 400-micron thick coronal slices. The coordinates for the hippocampus were: AP (antero-posterior)—3.8 mm posterior to Bregma, and L (lateral)—±1.8 to ±2.2 mm. The coordinates for the prefrontal cortex were: AP—+3.8, L—±0.6 and V—3.8 (Paxinos & Watson, 2005). The slices were post-fixed in 1% osmium tetroxide for 2 hr. The regions of interest were pyramidal cell layer + stratum oriens in the CA1 and CA3 areas of the hippocampus and deep layers in the medial prefrontal cortex. These regions were identified with an optical microscope Leica MM AF, cut out from the coronal slices, dehydrated in graded series of ethanol and acetone and embedded in Araldite. From Araldite blocks, 70–75 nm thick sections were prepared with an ultramicrotome Leica EM UC7. The sections were picked up on 200-mesh copper grids, double-stained with uranyl acetate and lead citrate and examined with JEM 1400 (JEOL, Japan). From each region of each rat, every fifth section—20 sections from the CA1, 20 sections from CA3 and 20 sections from the prefrontal cortex were evaluated and the ultrastructure of principal cells, interneurons, glia, synapses and pericapillary space was described.

2.3.1 | Quantitative analysis

On EM micrographs taken from CA1 area of PPA- and PBS-treated brain quantitative analysis of different parameters of axodendritic synapses: the area of presynaptic terminals, total number of synaptic vesicles, the numbers of docking, ready and resting vesicles, the number of presynaptic mitochondria and cristae, the number of symmetrical and asymmetrical synapses, the number of presynaptic terminals without active zone and the main morphological parameters of the porosome complex—the diameter of the opening and the depth of the cup-shaped structure were measured. The technique was partly described in the previous study (Lobzhanidze et al., 2019). Briefly, to measure the surface area of axon terminals, the number of presynaptic mitochondria, the number of symmetrical and asymmetrical synapses and the number of axon terminals without the active zone, 350 presynaptic terminals from PPA-treated brains and the same number of axon terminals from PBS-treated brains (70 terminals from each animal) were evaluated.

The tracings of axon terminals were scanned, using the scan plug-in for Adobe Photoshop CS3 and saved as 150 dpi tiff files. The scans were imported into ImageJ software (version 1.44, The National Institute of Mental Health). The counting of synaptic vesicles (total number and the number of vesicles in different pools) and mitochondrial cristae was performed on 150 axon profiles from PPA- treated rats and 150 profiles from PBS-treated rats (30 profiles from each animal), using Photoshop.

The counting of synaptic vesicles (total number and the number of vesicles in different pools) and mitochondrial cristae was performed on 150 axon profiles from PPA-treated rats and 150 profiles from PBS-treated rats rats (30 profiles from each animal), using Photoshop. For this purpose, the images of the axon terminals were enlarged onto the computer screen and each vesicle and cristae were sequentially marked, using the brush tool. For the measurement of opening diameter and the depth of porosome complex—secretory machinery of cell (Cho et al., 2004; Jena, 2015), totally 201 synaptic profiles were observed: $n = 101$ in PPA-treated brain and $n = 100$—in PBS-treated brain. Quantitative data were analysed using the Minitab software. To determine whether PPA affects above-mentioned parameters, two-sample t test was used. A p-value of less than .05 was considered as statistically significant. The results were presented as a mean ± *SEM*.

3 | RESULTS

3.1 | Behavioural experiments

3.1.1 | The assessment of spatial memory in MWM

As depicted in Figure 4, in comparing with the first trial, during the second trial the PBS- and PPA-treated animals needed significantly less time to find the platform (39.39 ± 2.7 vs. 21.9 ± 3.5; $p = .001$; 45.9 ± 3.4 vs. 27.5 ± 4.1; $p = .002$ correspondingly). Thus, both groups were capable of learning the location of the platform.

During the retrieval phase, the comparison of the behaviour between PBS- and PPA-treated rats in the SW quadrant (where during the first and second trials the platform was located) was made. The analysis revealed that there was no statistically significant difference between these two groups neither 30 min nor 24 hr after the acquisition phase (25.05 ± 2.7 vs. 20.12 ± 1.1, $p = .109$; 15.29 ± 2.0 vs. 15.04 ± 1.4, $p = .918$ correspondingly). It means that both groups in both cases spent approximately the same time in the SW quadrant, hence their memory was not affected by PPA.

Also, the comparison of the behaviour of PBS-treated rats in the SW quadrant and its opposite (NE) one was made. The analysis revealed that the rats spent significantly less time in quadrant NE 30 min after the acquisition phase (25.05 ± 2.7 vs. 6.60 ± 1.0; $p = .000$). The same was true with PPA-treated (20.12 ± 1.1 vs. 10.09 ± 1.2; $p = .000$). 24 hr after the acquisition phase, the comparison of the behaviour of PBS-treated

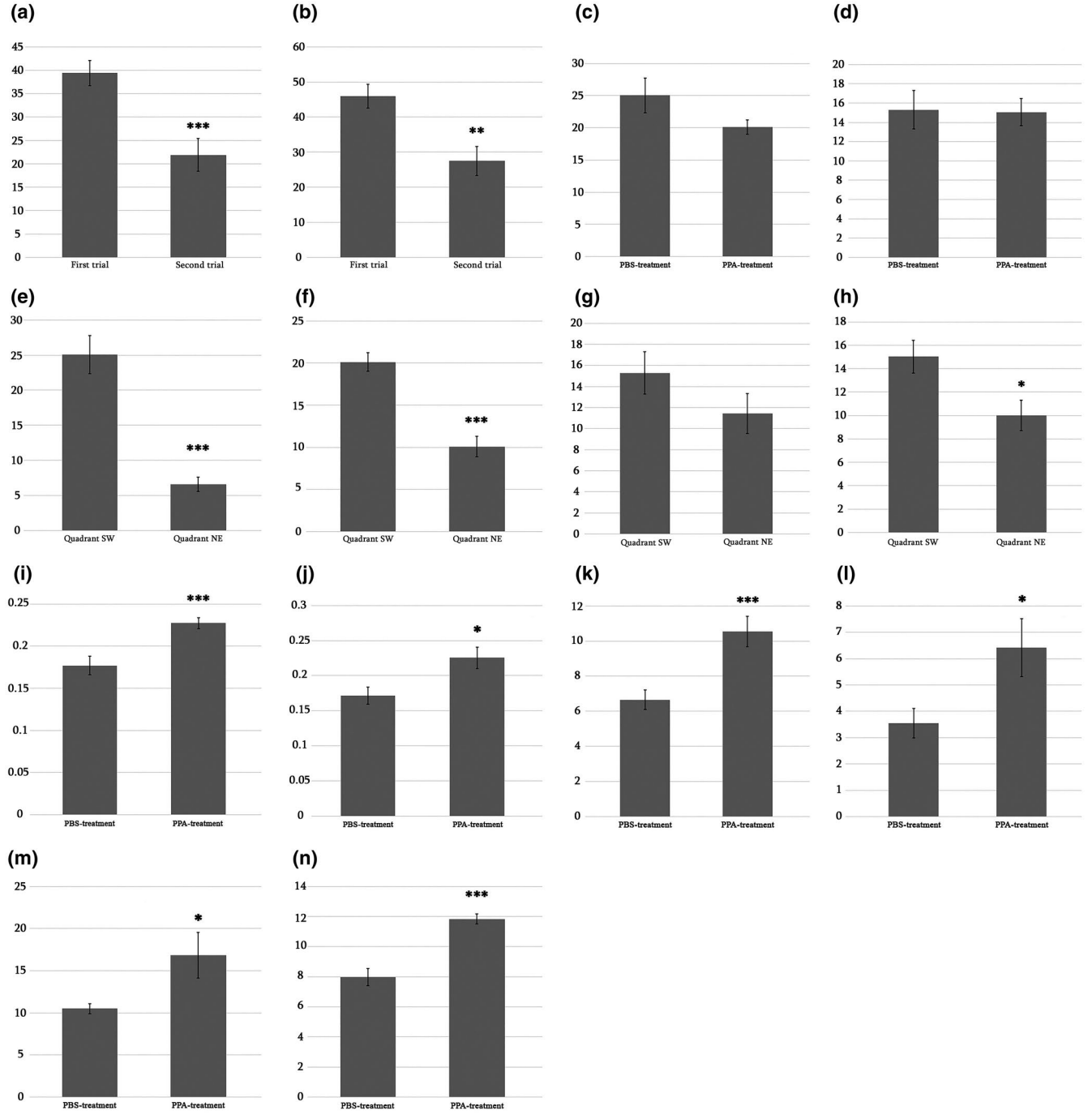

FIGURE 4 The assessment of reference memory in MWM. (a) Time needed to find the platform by PBS-treated rats during first and second trials, (b) Time needed to find the platform by PPA-treated rats during first and second trials, (c) Time spent by PBS- and PPA-treated rats in SW (target) quadrant 30 min after acquisition phase, (d) Time spent by PBS- and PPA-treated rats in SW (target) quadrant 24 hr after acquisition phase, (e) Time spent by PBS-treated rats in SW (target) and opposite quadrants 30 min after acquisition phase, (f) Time spent by PPA-treated rats in SW (target) and opposite quadrants 30 min after acquisition phase, (g) Time spent by PBS-treated rats in SW (target) and opposite quadrants 24 hr after acquisition phase, (h) Time spent by PPA-treated rats in SW (target) and opposite quadrants 24 hr after acquisition phase, (i) Swimming velocity of PBS- and PPA-treated rats during first trial, (j) Swimming velocity of PBS- and PPA-treated rats during second trial, (k) Distance covered by PBS- and PPA-treated rats during first trials, (l) Distance covered by PBS- and PPA-treated rats during second trials, (m) Distance covered by PBS- and PPA-treated rats 30 min after acquisition phase, (n) Distance covered by PBS- and PPA-treated rats 24 hr after acquisition phase. The data demonstrate significant effect of PPA on rat locomotor activity and no effect of PPA on memory

rats in SW quadrant and its opposite (NE) one revealed that the rats spent lesser time in quadrant NE, but the difference was not significant (15.29 ± 2.0 vs. 11.42 ± 1.9; $p = .179$). As to PPA-treated rats, they spent significantly less time in quadrant NE (15.04 ± 1.4 vs. 10.00 ± 1.3; $p = .014$). Therefore, no effect of PPA on short-and long-term memory in MWM was detected.

As the next step, the velocity of animal swimming was compared. During both trials, PPA-treated rats swam faster than PBS-treated rats (First trial: 0.1768 ± 0.011 vs. 0.2273 ± 0.0067, $p = .001$; Second trial: 0.1715 ± 0.012 vs. 0.2254 ± 0.015; $p = .01$).

Finally, the distance covered by PBS- and PPA-treated rats was evaluated. During both trials, this parameter was higher in PPA-treated rats (First trial: 6.65 ± 0.55 vs. 10.54 ± 0.87; $p = .001$; Second trial: 3.54 ± 0.56 vs. 6.42 ± 1.1; $p = .028$). The same was 30 min and 24 hr after the acquisition phase (After 30 min: 10.49 ± 0.58 vs. 16.83 ± 2.7; $p = .040$; After 24 hr—7.96 ± 0.57 vs. 11.84 ± 0.33; $p = .000$). These data indicate that the PPA treatment increases the motor activity of rats in MWM (Figure 4). 10.54 ± 0.87; $p = .001$; Second trial: 3.54 ± 0.56 versus 6.42 ± 1.1; $p = .028$). The same was 30 min and 24 hr after the second trial (After 30 min: 10.49 ± 0.58 vs. 16.83 ± 2.7; $p = .040$; After 24 hr—7.96 ± 0.57 vs. 11.84 ± 0.33; $p = .000$). These data indicate that the PPA treatment increases the motor activity of rats in MWM (Figure 4).

3.1.2 | The assessment of the process of learning in MBM

As showed in Figure 5, PPA and PBS-treated rats showed no difference between the number of entries in "closed" arms (0.406778 ± 0.151889 vs. 0.238189 ± 0.128444; $p > .05$ respectively). Such data suggest no significant effect of PPA on learning ability in this paradigm. However, PPA-treated rats moved significantly slower than PBS-treated rats and needed more time to cover the distance between starting point to nest-cage (48.34444 ± 11.6 vs. 229.3222 ± 43.44444, $p < .05$ respectively).

3.1.3 | The assessment of locomotor activities in OF

As depicted in Figure 6, in comparing with PBS-treated rats, PPA-treated rats showed a significant decrease of the locomotor activity (35.5 ± 3.3 vs. 23.6 ± 3.7; $p = .028$). Therefore, in OF the decrease of locomotor activity as a result of PPA-treatment was shown.

3.1.4 | Social tendency

The data showed that PPA-treated rats spent significantly less time (in seconds) with unfamiliar conspecific rats than did their PBS-treated counterparts (459 ± 52 vs. 236 ± 70; $p = .019$). No difference was found in the number of visits to the unfamiliar rat between PPA and PBS-treated animals (5.17 ± 1.2 vs. 3.92 ± 1.2; $p = .478$). As for the unfamiliar object, no difference between PPA and PBS-treated rats was found neither regarding the number of visits to this object (1.58 ± 0.76 vs. 2.83 ± 0.98; $p = .325$), nor in total time spent near it (16.1 ± 10 vs. 29.5 ± 9.6; $p = .35$) (Figure 7). The

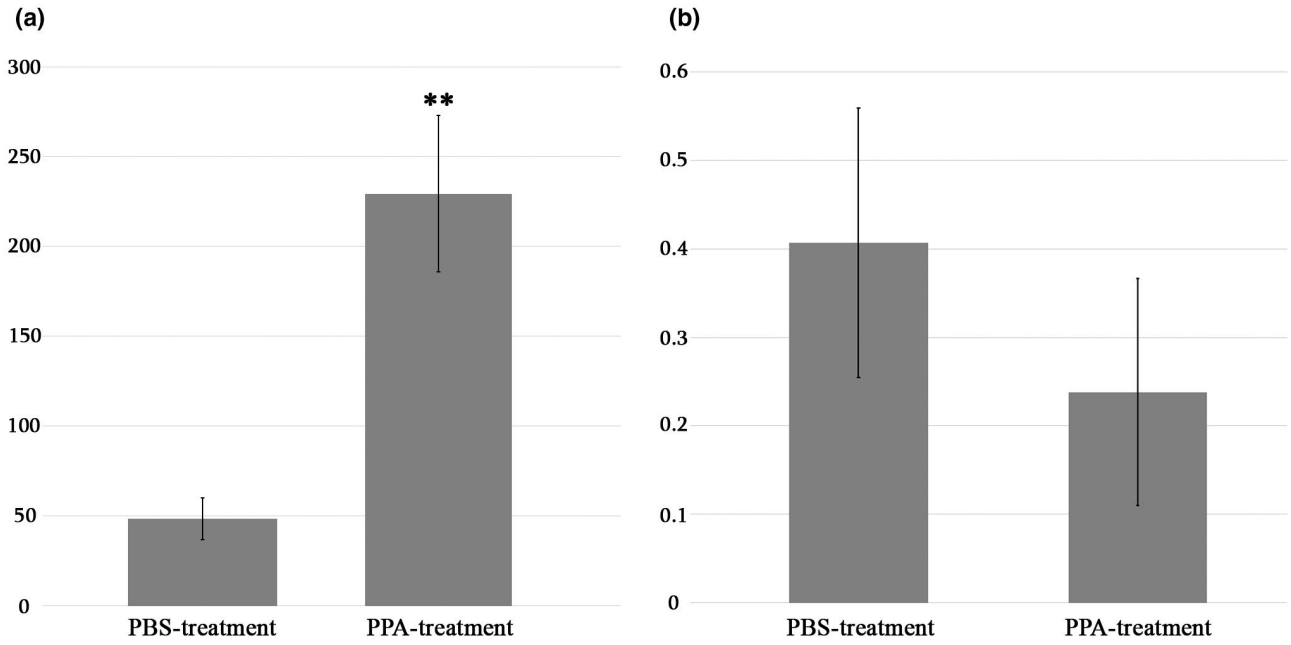

FIGURE 5 The assessment of learning in MBM. The average of the ten days measurement of behavioural activities of PPA and PBS-exposed adolescent male rats in MBM. (a) The time needed to pass the MBM. (b) The number of entries into "closed" arms (errors). Bars represent means ± *SEM*, $n = 12$. The data indicate that PPA-treated rats move slowly than PBS-treated rats and need more time to cover the distance. No PPA effect on the process of learning was shown

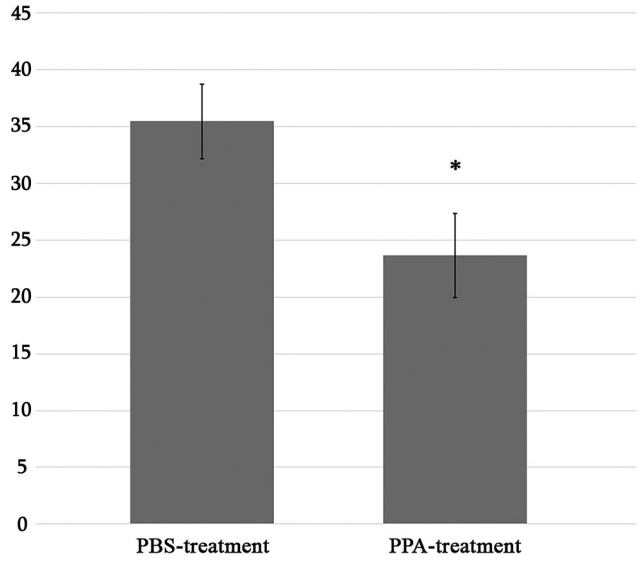

FIGURE 6 The assessment of locomotor activity in OF. PPA treated rats show significant decrease of locomotor activity when testing in OF

data indicate that PPA treatment produces a decrease of social motivation but the asocial motivation remains the same.

3.2 | EM analysis of the effect of IP PPA administration on the hippocampus and prefrontal cortex

In comparing with PBS-treated brain, the ultrastructure of PPA-treated brain was slightly altered. Mild/moderate changes were mostly observed in the hippocampus, especially in the CA1 area. About 4% of observed large neurons of this area (with great probability—large pyramidal cells) had highly osmiophilic cytoplasm, dark shapeless nucleus, irregular boundaries and pathologically modified organelles. Such "cells" were surrounded by activated astrocytes or microglia (Figure 8a,b). Approximately the same number of large pyramidal cells revealed the signs of chromatolysis: the cytoplasm density was low, the organelles were few, several organelles were pathologically modified (Figure 8c,e,f).

Commonly, in large neurons, multivesicular bodies or peroxisomes with crystalline inclusions were observed (Figure 8e,f). Mitochondria were mainly unchanged (unless mitochondria in degenerated or chromatolytic cells), but showed remarkable variations in sizes and forms. The majority of synapses had normal ultrastructure; however, few atypically enlarged presynaptic terminals with single synaptic vesicles and short active zone were also observed (Figure 8i). The activated astrocytes and microglia were comparatively common. Some astrocytes, including pericapillary forms, contained different pathological inclusions or multivesicular bodies (Figure 8d,g,h). No noticeable changes in the endovasculature were detected.

In comparing with the CA1 area, the CA3 region was more preserved. With less frequency dark neurons or the neurons with chromatolysis, destructed organelles, multivesicular bodies, as well as activated or modified astrocytes and microglia were observed. In the medial prefrontal cortex, only single superficial alterations were seen.

3.2.1 | Quantitative analysis of synaptic parameters

In comparing with PBS-treated rats (Figure 9), in the CA1 area of PPA-treated brain significant decrease of total number of synaptic vesicles (165 ± 27 vs. 68.4 ± 11; $p = .003$), total number of presynaptic mitochondria (4.22 ± 0.62 vs. 1.33 ± 0.44; $p = .001$), and the number of presynaptic terminals without active zone (4.80 ± 0.77 vs. 1.83 ± 0.39; $p = .002$) was detected. No difference was observed regarding the number of synaptic vesicles from different vesicle pools (Docked: 4.22 ± 0.82 vs. 4.00 ± 0.61; $p = .835$. Ready: 12.6 ± 2.7 vs. 9.1 ± 2.2; $p = .311$. Resting: 23.3 ± 4.9 vs. 23.5 ± 5.8; $p = .976$), area of presynaptic terminals (2,274,884 ± 360,407 vs. 1,453,679 ± 280,233; $p = .081$), total number of symmetrical and asymmetrical synapses (0.64 ± 0.17 vs. 0.661 ± 0.13; $p = .913$), the number of symmetrical synapses (1.14 ± 0.27 vs. 1.21 ± 0.21; $p = .856$), the number of asymmetrical synapses (0.131 ± 0.13 vs.0.133 ± 0.063; $p = .986$) and the number of mitochondrial cristae in presynaptic terminals (3.63 ± 0.31 vs. 3.49 ± 0.23; $p = .720$) (Figure 10).

4 | DISCUSSION

A number of studies point that chronic intracerebroventricular, i.p., or intracutaneous treatment of rodents with different doses of PPA (4.0 µl, 250 and 500 mg/kg respectively) produces behavioural, immmunocytochemical and biochemical changes that are reminiscent with those in ASD (Choi et al., 2018; Foley, MacFabe, Vaz, Ossenkopp, & Kavaliers, 2014; MacFabe et al., 2007; Ossenkopp et al., 2012; Shultz et al., 2015). Unlike these studies, in the present investigation, the rats received a *single i.p. injection of relatively low dose of PPA (175 mg/kg).* The results show that such regime of treatment is also capable of altering social behaviour and locomotion. Furthermore, this exposure is capable of producing measurable changes on the ultrastructure and presynaptic architecture of the axodendritic synapses of the hippocampal CA1 area, which plays a key role in memory, social function and cognition (Eichenbaum, 2017; Matta, Tiessen, & Choleris, 2017; Okuyama, 2018; Rolls & Wirth, 2018).

Social impairment is one of the key characteristics of ASD and PPA treatments (Foley et al., 2014; MacFabe et al., 2007;

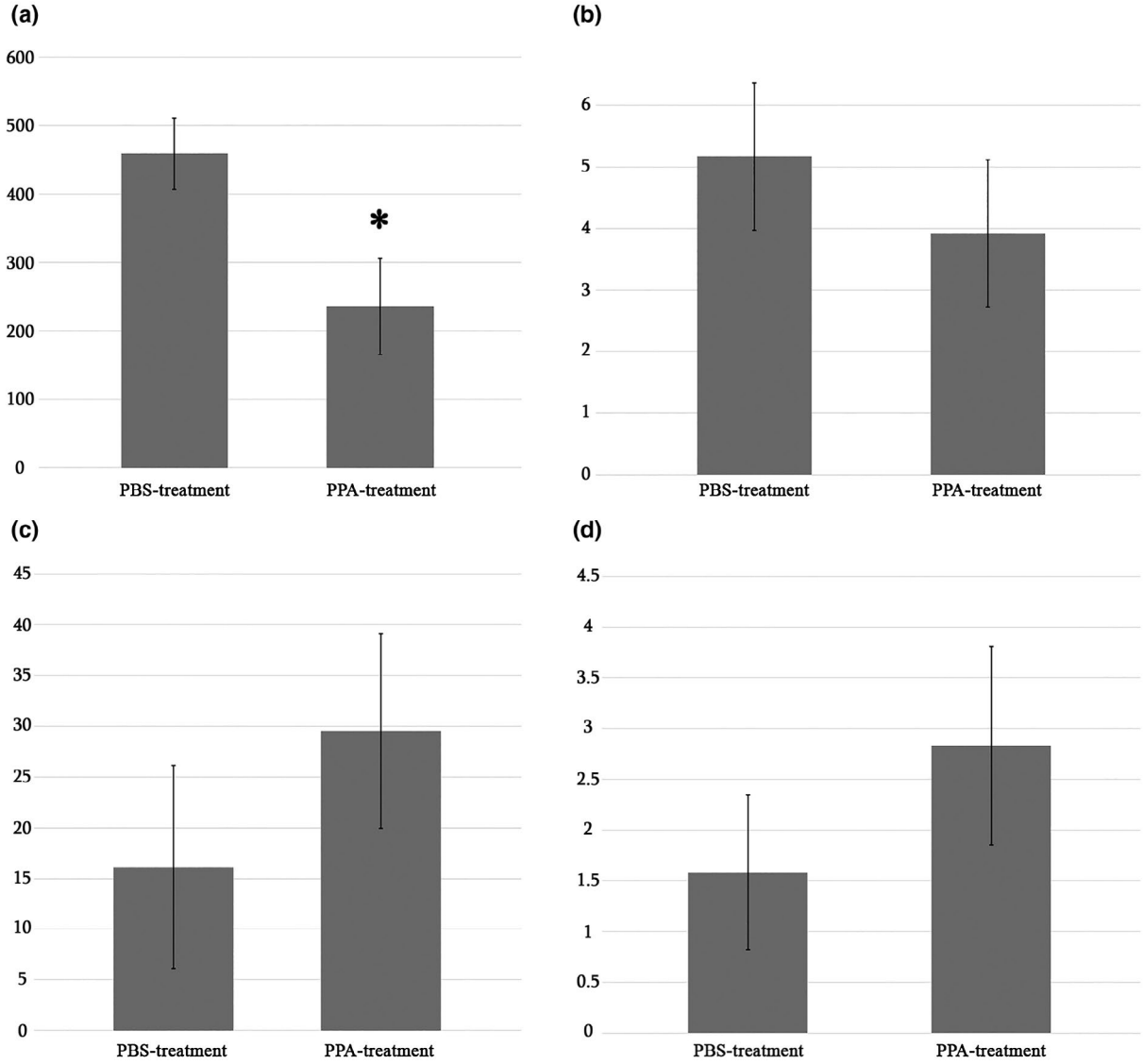

FIGURE 7 The assessment of social behavior. The following data are presented. (a) The total time spent in close vicinity with unfamiliar rat (social zone). (b) The number of visits to unfamiliar rat (social zone). (c) The total time spent in close vicinity with unfamiliar object (asocial zone), d) the number of visits to unfamiliar object (asocial zone). *$p < .05$. The data indicate that PPA-treated rats spend significantly less time with unfamiliar rat. Therefore, PPA treatment produces the decrease of social motivation, while asocial motivation remains the same

Shams et al., 2019; Shultz et al., 2008). Previously, using the same regime of PPA injection as in the present study, we reported measurable social deficits 120 min after the PPA treatment (Lobzhanidze et al., 2019). Now we show that this deficit, in couple with unchanged asocial motivation, remains present even 5 days after the treatment. Therefore, this effect of PPA was relatively persistent.

The changes in locomotor activity are another feature of PPA treatment. In the present study, the dual character of locomotor changes was observed: in MBM and OF, the reduced locomotor activity was revealed, while, in contrast, the results of the MWM test show an increased velocity of swimming. Several data point to such duality of the PPA effect. Thus, hypoactivity, hypotonia and abnormal motor movements are often detected in rodents chronically treated with PPA (Ossenkopp et al., 2012; Shams et al., 2019). In contrast, female offspring of rats prenatally exposed to PPA were significantly more active than vehicle-treated females (Foley et al., 2014). PPA-provoked hyperlocomotion was also described in a number of recent studies performed on young rats (Daghestani et al., 2017; Mirza & Sharma, 2018). Such contradictory results need further evaluation but may relate to the different behaviour paradigms, leading to increased anxiety-like behaviours via increased swimming, in the MWM, or decreased motor activity in the MBM and OF. They may also relate to findings in ASD patients, some of which often increase in solitude (decreased anxiety, increased repetitive behaviour) versus social interaction (increased anxiety).

In the present study, we did not observe the effect of PPA on the process of learning (MBM test) and reference memory

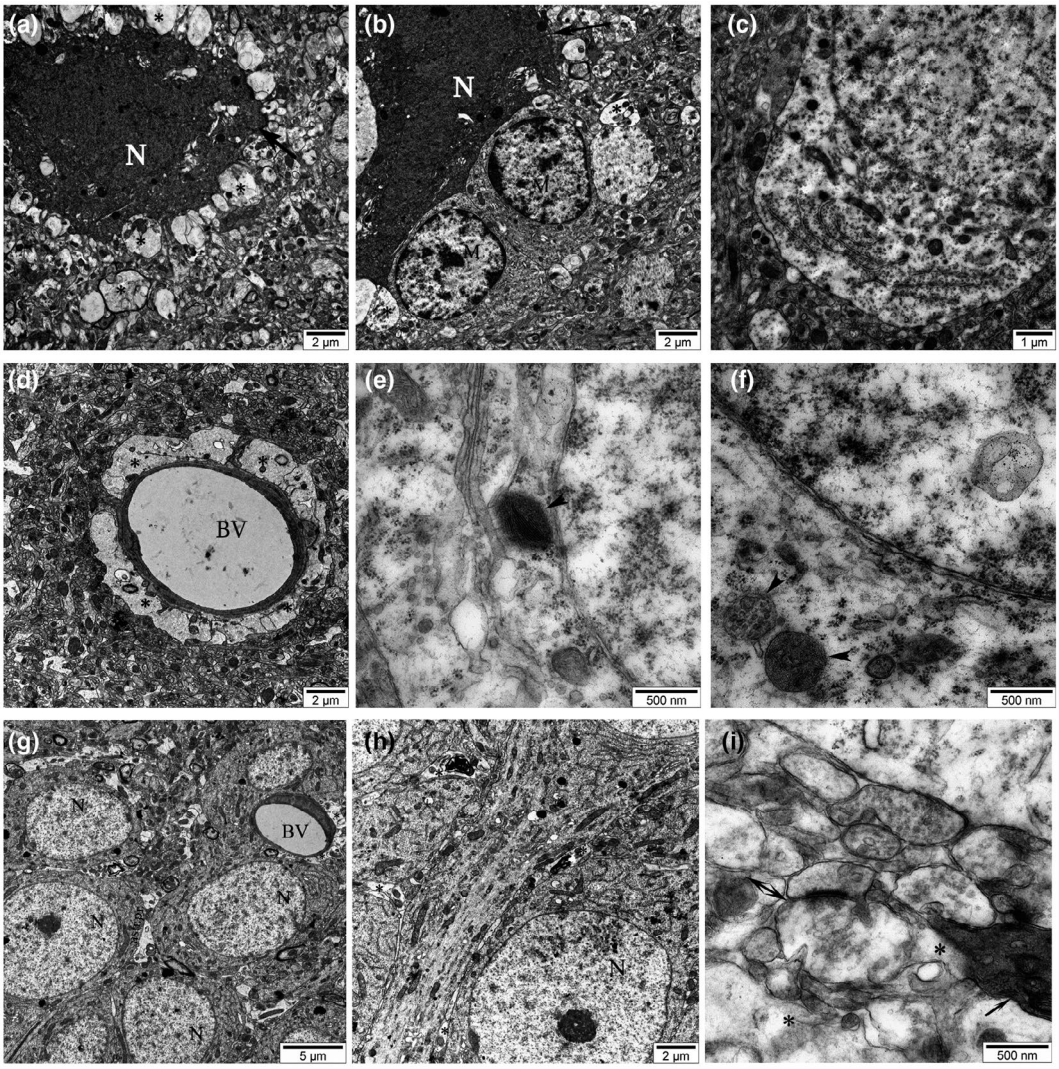

FIGURE 8 Representative transmission electron microscopy images of neurons, glia and capillaries from CA1 area of the hippocampus. (a) Damaged necrotic neuron surrounded with swollen astrocytic processes. (b) Damaged necrotic neuron and microglial cells. (c) Early stage of chromatolysis in neuronal cytoplasm. (d) Blood vessel surrounded with swollen astrocytic processes. (e) The part of neuron with peroxisome in close vicinity from nucleus. (f) Multivesicular bodies and secondary lysosome in neuronal cytoplasm. (g) Normal looking neurons and small uundamaged capillary with moderately swollen astrocytic process. (h) Dendrite with well seen microtubules, several lysosomes, cisterns of endoplsmic reticulum and mitochondria. In neuropil moderately swollen astrocytic processes with myeline insertions were observed. (i) Axo-dendritic synapse with few synaptic vesicles and short asymmetric active zone. The presynaptic terminal is moderately swollen. In neuropil dark process of degenerated cell is observed. Swollen astrocytes are concentrated in close vicinity of terminal and degenerated process (N—neuron; BV—blood vessel; M—microglial cell; per—peroxisome; MVB—multivesicular bodies; arrows—necrotic neurons; arrowhead—secondary lysosome; asterisk—swollen astrocytic processes)

(MWM test). However, numerous data, including ours, indicate altered cognition as a result of PPA chronic treatment (Choi et al., 2018; Foley et al., 2014; MacFabe et al., 2011; Mepham et al., 2019). Such discrepancy of results may have several causes, for example, the regime of treatment (single injection of low dose vs. chronic treatment with relatively high dose), the difference in animal strain (in previous studies Long-Evans rats were used, while present research was performed on Wistar rats), the age of animals (earlier mostly adolescent rats in the age of P 41 ± 4 were used, while in the present study the animals were younger, therefore, had higher compensatory properties), or the difference between times of testing. Finally, it should be noted the presence of only a few pathological modifications in the hippocampus and the absence of such changes in the prefrontal cortex. The dependence of spatial memory on hippocampal integrity is well known (Bast, Pezze, & McGarrity, 2017; Montagrin, Saiote, & Schiller, 2018; Schafer & Schiller 2019). However, the memory is impaired only after bilateral dorsal hippocampal lesions that encompass 30% or more of the hippocampus total volume (Broadbent, Squire, & Clark, 2004; Colombo, Wetsel, & Gallagher, 1997). Moreover, besides the hippocampus,

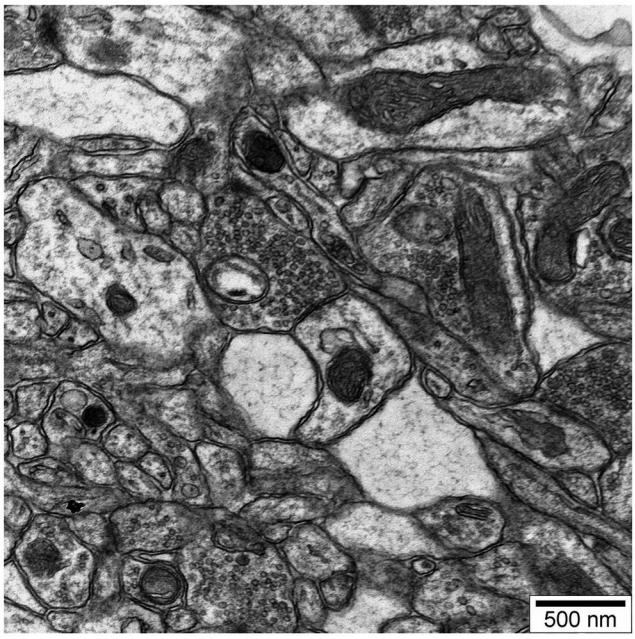

FIGURE 9 Representative figure of a control (untreated) brain tissue demonstrating presynaptic terminals with normal number of vesicles

spatial memory functions are cortically supported: the tasks associated with the hippocampus can be mediated by external structures, such as the prefrontal region (Anderson, Bunce, & Barbas, 2016; Griffin, 2015). Here we show that PPA affects the much smaller part of the hippocampus, while the prefrontal cortex retained almost normal ultrastructure. It is also notable that during adolescence, molecular composition and synaptic function of both regions still undergo developmental modifications, indicating to a high level of neuroplasticity of these areas in response to physiological and pathological conditions (Counotte et al., 2010; Gómez & Edgin, 2016; Johnson-Venkatesh, Khan, Murphy, Sutton, & Umemori, 2015).

Our study revealed relatively high susceptibility of the CA1 hippocampal area to PPA treatment. Molecular, histological and biochemical data also point out the altered CA1 area in PPA and other autism models (Dahlhaus et al., 2010; Guang et al., 2018; MacFabe et al., 2007). The special vulnerability of this region to various neuropathological conditions (epilepsy, depression, encephalitis, stroke, limbic encephalitis, hypoglycaemic encephalopathy, multiple sclerosis, transient global amnesia, Alzheimer disease, etc.) is well known (Liang et al., 2013; Medvedeva, Ji, Yin, & Weiss, 2017). The basis of such susceptibility is still obscure but may include genetically determined glutamate-dependent and calcium-mediated mechanisms of neuronal excitability and oxidative stress (Bartsch et al., 2015). Under EM, we detected few dark formless "cells." Based on the size and ultrastructure of these "cells," we propose that they should be apoptotic residues of large pyramidal neurons, which are the most populous excitatory cell type in the hippocampus and usually contain the excitatory transmitter glutamate. Other large modified cells represent chromatolitic neurons. Chromatolysis is often considered as the preapoptotic stage or specific apoptotic process, where the Nissl substance disintegrates. Therefore, present data coincide with the results of our previous and other studies, where the proapoptotic effect of high dose of PPA was proposed (Choi et al., 2018; El-Ansary et al., 2018; Lobzhanidze et al., 2019). CA1 is the first region in the hippocampus circuit, from which a major output pathway projecs to enthorinal cortex. The degeneration of only a few number of large pyramidal (projection) neurons point to a superficial/mild effect of PPA on the hippocampal networks, which may not significantly change their functions. The identification of these cell types is the subject of further study.

Of particular interest are the results of EM quantitative data, indicating to a significant decrease in the total number of axodendritic synapses, synaptic vesicles and presynaptic mitochondria. Such results are reminiscent with our previous studies, where the alterations of synapse associated proteins, including CREB, neurexins and neuroligins, following PPA chronic central administration in rat brain (MacFabe, 2012, 2015), rat PC12 cells (Nankova et al., 2014), human neurotypical and ASD cell lines (Frye et al., 2016, 2017; MacFabe, 2015) and recently, human neural progenitor cells (Yang et al., Neurochemistry, accepted for publication). Because in our material the changes in the vesicle number and"active" presynaptic profiles were accompanied with the decrease of the number of presynaptic mitochondria (which should reflect the difficulties in energy synthesis), we suggest that such changes could be related more with the decrease of neurotransmitter production rather than the increased release of neurotransmitters.

Despite detailed analysis, we did not observe significant changes in the fine morphology of porosome complex—secretory machinery of cell, directly related to the neurotransmission (Cho et al., 2004; Jena, 2015). Therefore, we do not exclude that fine alterations take place before synaptic vesicles dock with the synaptic membrane. Our ongoing studies (such as analysis of the area of synaptic vesicles from different pools) address this issue. In this research we were focused only on axodendritic synapses; however, it will be important to perform a comparative study of axodendritic and axosomatic synapses, as well as the chemistry of neurons vulnerable to the PPA treatment, in order to assess which types of cells and synaptic contacts are more susceptible.

Interesting EM observation was the increased number of peroxisomes and endosomes in the CA1 area of the PPA-treated brain. Such an ultrastructural feature usually indicates to the increased endocytic activity and the activation of reactive oxygen species detoxification system (Mirza & Sharma, 2018; Patak, Zhang-James, & Faraone, 2016; Von

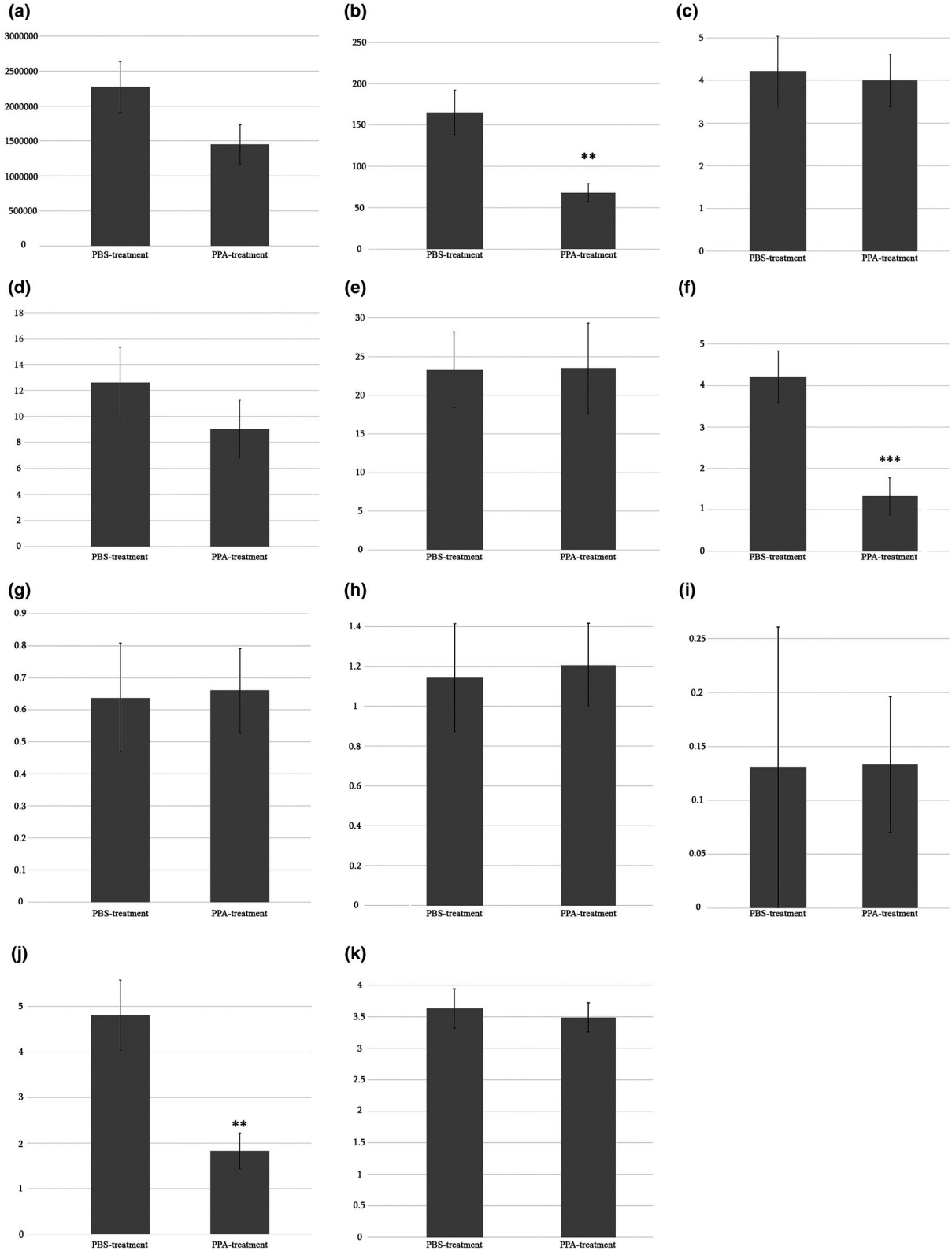

FIGURE 10 The results of quantitative electron microscopic analysis of different parameters of synapses in PPA- and PBS-treated rats. (a) Area of presynaptic terminals. (b) Total number of synaptic vesicles. (c) Number of docking vesicles. (d) Number of ready vesicles. (e) Number of resting vesicles. (f) Number of presynaptic mitochondria. (g) Total number of asymmimetrical and symmetrical synapses. (h) Number of presynaptic terminals with symmetrical zone. (i) Number of presynaptic terminals with active asymmetric zone. (j) Number of presynaptic terminals without active zone. (k) Number of mitochondrial cristae in presynaptic terminals

Bartheld & Altick, 2011). The data are reminiscent with our previous biochemical results, according which chronic ICV administration of PPA provokes the aberrations in lipid metabolism known to affect membrane fluidity, peroxisomal function, gap junction, coupling capacity and neuroinflammation, all of which may be associated with ASD pathogenesis (MacFabe et al., 2007; Thomas et al., 2012). Of note, a recent study found a selective modulator of peroxisome proliferator-activated receptor protects PPA-induced autism-like phenotype in rats (Mirza & Sharma, 2018). Interestingly, peroxisomes and endosomes were rare in the amygdala of rats treated with PPA treated in the same manner to this present study (Lobzhanidze et al., 2019).

Autism is often associated with mitochondrial disease and abnormalities in energy generation (Bu et al., 2017; Hollis, Kanellopoulos, & Bagni, 2017). Chronic injection of PPA is known to provoke aquired mitochondrial dysfunctions, the level of which depends on the character of treatment (Choi et al., 2018; Frye et al., 2016, 2017; MacFabe, 2015). In the present study, mitochondrial pathologies were observed only in degenerated and chromatolitic neurons. Quantitative analysis of presynaptic mitochondrial cristae also did not reveal the difference between PPA- and PBS-treated brains. However, structural diversities of "normal" mitochondria were common. Three-dimensional reconstruction of mitochondria, the measurements of their volume, the studies of electron transport chain complexes and lipid metabolism should provide detailed information regarding the dual character of mitochondrial changes.

Activation of astrocytes and microglia is common in the ASD autopsy material (Menassa, Sloan, & Chance, 2017; Pardo, Vargas, & Zimmerman, 2005; Vargas, Nascimbene, Krishnan, Zimmerman, & Pardo, 2005) and represents the important feature of PPA action (Choi et al., 2018; De Almeida, Funchal, Gottfriedl, Wajner, & Pessoa-Pureur, 2006; MacFabe, 2015). It is proposed, that in normal and pathological conditions PPA and butyrate increase neuronal histone acetylation, but are metabolized mainly in glia (Nguyen et al., 2007). Glial effects may be related to the presence of free fatty acid receptors on brain macro- and microglia (Erny, Hrabe de Angelis, & Prinz, 2017; Park, Wang, Wu, Mao-Draayer, & Kim, 2019). Our EM studies revealed that the rapid activation of glia has developed even after a single injection of a relatively low dose of PPA (Lobzhanidze et al., 2019). Such fast-paced changes, which should be related to inflammatory processes and high degree of glial cell plasticity, demand further investigation.

In the PPA-treated brain, we did not observe modifications in vascular endothelial cells and gap-junctions in our treatment regime. Gap junctions, formed from connexins, are important structural component of cell-cell communications, the changes of which, together with the alterations in membrane fluidity, peroxisomal function and neuroinflammation, are considered as a direct result of the aberrant lipid metabolism provoked by PPA-treatment (Frye et al., 2016; MacFabe, 2012; Thomas et al., 2012). We propose that the EM immunocytochemical study of connexin proteins in the PPA-treated brain may provide deeper information regarding gap-junctions possible changes.

4.1 | Summary

The research has shown that even single treatment of adolescent male rats with relatively low single i.p. dose of PPA leads to enduring impairments to social interaction and locomotor activity, coupled with fine modifications in the CA1 area. These changes may also be dynamic and await further study. This is in accordance with our previous data where the reversible character of PPA-provoked alterations was proposed (MacFabe, 2015; Mepham et al., 2019). It should be noted that the PPA action might be extremely complex and potentially reinforcing, and include cell-cell interaction, immunity, lipid changes, mitochondrial/energy metabolism and gene expression, in many organ systems including the brain. At this preliminary stage, we do not know the exact molecular mechanisms, permanence, dose and time sensitivity, other anatomical regions, sex differences or critical developmental windows. These await further study. EM should provide high-resolution information regarding the effect of PPA and other short chain fatty acids.

In conclusion, this study is consistent with our proposal that PPA and related short chain fatty acid bacterial metabolites may provide a potential mechanism, where the microbiome dynamically modulates brain function and behaviour at various levels, including synapse architecture, throughout the lifecycle in health and disease, and further supports the validity of the PPA model of ASD (Lobzhanidze et al., 2019; MacFabe, 2015).

CONFLICT OF INTEREST

The authors declare no competing interests.

ETHICS

The experiments were conducted in accordance with European Union Directive on the protection of animals used for scientific research. The Committee of Animal Care at the Ivane Beritashvili Center of Experimental Biomedicine and Committee on Ethics at the Ilia State University approved all procedures.

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DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

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SUPPORTING INFORMATION

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