

Altered brain phospholipid and acylcarnitine profiles in propionic acid infused rodents: further development of a potential model of autism spectrum disorders

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Abstract

Recent studies have demonstrated intraventricular infusions of propionic acid (PPA) a dietary and enteric short-chain fatty acid can produce brain and behavioral changes similar to those observed in autism spectrum disorder (ASD). The effects of PPA were further evaluated to determine if there are any alterations in brain lipids associated with the ASD-like behavioral changes observed following intermittent intraventricular infusions of PPA, the related enteric metabolite butyric acid (BUT) or phosphate-buffered saline vehicle. Both PPA and BUT produced significant increases ($p < 0.001$) in locomotor activity (total distance travelled and stereotypy). PPA and to a lesser extent BUT infusions decreased the levels of total monounsaturates, total $\omega 6$ fatty acids, total

phosphatidylethanolamine plasmalogens, the ratio of $\omega 6 : \omega 3$ and elevated the levels of total saturates in separated phospholipid species. In addition, total acylcarnitines, total long-chain (C12–C24) acylcarnitines, total short-chain (C2 to C9) acylcarnitines, and the ratio of bound to free carnitine were increased following infusions with PPA and BUT. These results provide evidence of a relationship between changes in brain lipid profiles and the occurrence of ASD-like behaviors using the autism rodent model. We propose that altered brain fatty acid metabolism may contribute to ASD.

Keywords: carnitine, clostridia, gap junctions, lipids, mitochondria, movement disorder, short-chain fatty acids. *J. Neurochem.* (2010) **113**, 515–529.

Autism spectrum disorder (ASD) is a family of neurodevelopmental disorders characterized by restrictive interests, repetitive behaviors, deficits in social understanding and interactions, impairments in language development and communications skills. Associated symptoms also include self-injurious behavior, aggression and epilepsy (Amminger *et al.* 2007). During the last decade, ASD cases in North America indicate a dramatic increase with a prevalence of

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Abbreviations used: ASD, autism spectrum disorder; BUT, butyric acid; CL, cardiolipin; DMA, dimethyl acetal; ESI-MS, electrospray ionization mass spectrometry; FAME, fatty acid methyl ester; FID, flame ionization detection; GC/MS, gas chromatography/mass spectrometry; ICV, intracerebroventricular; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PPA, propionic acid; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SM, sphingomyelin.

Received October 21, 2009; revised manuscript received January 18, 2010; Accepted January 19, 2010.

one in every 150 children under the age of 5 years (Bertrand *et al.* 2001; Herbert *et al.* 2006).

Evidence exists for a strong genetic contribution to ASD. However, the lack of complete concordance as well as variances in the severity of autism in monozygotic twins (Hu *et al.* 2006) indicate that the recent rates of ASD increases cannot be explained entirely by genetic links or improvement in diagnostic techniques, and suggest that environmental factors must also be involved (Bell *et al.* 2000). An emerging possibility is that ASD is a systematic encephalopathic condition involving immune, digestive and metabolic dysfunction exacerbated by environmental triggers in genetically sensitive subpopulations (Ashwood and Van de Water 2004; Chauhan and Chauhan 2006; Herbert *et al.* 2006; James *et al.* 2006). A number of environmental agents have been proposed to contribute to the pathogenesis of ASD including pre- and postnatal exposure to ethanol (Arndt *et al.* 2005), valproic acid (Ingram *et al.* 2000), terbutaline (Zerrate *et al.* 2007), some metals and viral infections (Fatemi *et al.* 2000). In addition, anecdotal reports have indicated an induction or exacerbation of ASD symptoms following acute gastrointestinal abnormalities (Horvath *et al.* 1999), routine and antibiotic resistant pediatric infections (Finegold *et al.* 2002b; Fallon 2005), and ingestion of wheat or dairy-containing foods (Jyonouchi *et al.* 2002). Furthermore, in a subset of patients a general improvement in ASD symptoms can be observed following the elimination of these food products from the diet (Jyonouchi *et al.* 2002). Collectively, these observations have led to the development of a number of animal models to study how environmental agents may contribute to the pathogenesis of ASD (Ingram *et al.* 2000; Narita *et al.* 2002; Kamitani *et al.* 2003; Fatemi *et al.* 2005). One relatively new potential ASD model is the propionic acid (PPA) rodent model developed in our laboratory (MacFabe *et al.* 2007, 2008; Shultz *et al.* 2008, 2009). In this model, we hypothesize that PPA, and/or related enteric fatty acids, may be candidate environmental factors involved in the pathophysiology of some types of ASD.

PPA is a short-chain fatty acid and an intermediate of cellular metabolism. PPA is also a fermentation by-product of a subpopulation of opportunistic enteric bacteria (i.e. clostridia and propionibacteria) (Finegold *et al.* 2002a) and a common food preservative in refined wheat and dairy products (Brock and Buckel 2004). PPA, being a weak acid, exists in both aqueous and lipid soluble forms and can readily enter the systemic and CNS environments (e.g. blood-brain barrier) both passively, and actively, via specific monocarboxylate transporters (Maurer *et al.* 2004) and can cause health-related issues if the individual is unable to adequately metabolize short-chain fatty acids. PPA and other short-chain fatty acids (i.e. butyrate and acetate), affect diverse physiological processes such as cell signaling (Nakao *et al.* 1998), neurotransmitter synthesis and release (DeCastro *et al.* 2005), mitochondrial function (Wagner

et al. 2004), lipid metabolism (Hara *et al.* 1999), immune functions (Le Poul *et al.* 2003), gap junctional gating (Rorig *et al.* 1996), and modulation of gene expression through DNA methylation (Suzuki *et al.* 1996) and histone acetylation (Parab *et al.* 2007). In addition, subpopulations exist with organic acidemias, carnitine/B12 deficiency, pre- or postnatal exposure to ethanol or valproate that collectively have impairments in PPA metabolism and produce neurodevelopmental conditions similar to those observed in ASD (Coulter 1991; Calabrese and Rizza 1999; Wajner *et al.* 2004). Thus, PPA and related short-chain fatty acids could provide a link among the disparate behavioral, dietary, gut, metabolic and immune factors implicated in ASD.

Initial studies using this rodent model revealed that repeated brief infusions of PPA, but not control compounds (i.e. propanol) into the lateral cerebral ventricles of adult rats produced behavioral, biochemical, electrophysiological and neuropathological effects consistent with those seen in ASD (MacFabe *et al.* 2007, 2008; Shultz *et al.* 2008, 2009). These effects include reversible repetitive dystonic behaviors, hyperactivity, perseveration, turning, repulsion, caudate spiking, the progressive development of limbic kindled seizures and impairments in social behavior, suggesting that this compound has central effects. Examination of brain tissue from PPA-treated rats reveal innate neuroinflammatory responses (reactive astrogliosis and activated microglia), increases in oxidative stress markers, and a reduction of glutathione, a broad spectrum xenobiotic detoxifier, all consistent with findings from ASD patients (Chauhan *et al.* 2004; Vargas *et al.* 2004; Chauhan and Chauhan 2006; James *et al.* 2006). We are currently extending our investigations using this model to examine the potential role of lipid metabolism in ASD and related neurodevelopmental disorders.

Several reports have indicated that abnormal lipid metabolism may occur in a variety of neurodevelopmental disorders such as attention-deficit hyperactive disorder, dyslexia, dyspraxia and ASD (Bell *et al.* 2000; Vancassel *et al.* 2001; Richardson 2004; Amminger *et al.* 2007; Wiest *et al.* 2009). These abnormalities have been observed for fatty acids, phospholipases A2 and membrane phospholipids (both diacyl and plasmalogen forms). Limited studies and anecdotal reports have suggested improvements in ASD patients' general health and reduction in core symptoms following supplementation with polyunsaturated fatty acids (Bell *et al.* 2004; Amminger *et al.* 2007; Meguid *et al.* 2008). Lipids constitute 60% (dry weight) of human brains of which 20% is composed of polyunsaturated fatty acids (PUFA) (Meguid *et al.* 2008). PUFA are major lipid constituents of neuronal membranes and are absolutely essential for normal brain development and function. In addition, lipids modulate membrane fluidity, gene expression, cell signaling, neuronal excitability and provide a source of energy for the cells, particularly during early neurodevelopment (Kidd 2007; Wiest *et al.* 2009). Several recent studies have reported

abnormal acylcarnitine and phospholipid profiles in erythrocytes and plasma of autistic patients. Most notably, these studies report elevations in acylcarnitines (Clarke and Clark-Taylor 2004), phospholipase A2 activity (Bell *et al.* 2004) and saturated fatty acids (Bell *et al.* 2000, 2004; Pastural *et al.* 2009), accompanied by a decline in carnitine (Filipek *et al.* 2004), plasmalogens (Bell *et al.* 2000, 2004; Wiest *et al.* 2009), mono and polyunsaturated ($\omega 3 + \omega 6$) fatty acids (Bell *et al.* 2000, 2004; Vancassel *et al.* 2001; Bu *et al.* 2006; Meguid *et al.* 2008; Wiest *et al.* 2009). Conversely, others have reported elevations in the levels of plasmalogens (Pastural *et al.* 2009), mono- and polyunsaturated fatty acids (Bell *et al.* 2000; Sliwinski *et al.* 2006; Pastural *et al.* 2009; Wiest *et al.* 2009).

Collectively, these studies suggest altered lipid metabolism may occur in ASD. It is important, however, to note that all of these investigations report lipid alterations in blood obtained from ASD patients and not brain lipids. It could be argued that these alterations represent an epiphenomenon associated with a restricted diet or stress in ASD patients. We thus used our PPA rodent model to examine whether there is any evidence for alterations in brain lipids associated with the occurrence of ASD-like brain and behavioral changes shown to occur following intraventricular infusions with PPA in adult rats. Butyrate (BUT) infusions were also performed to determine if other gut-derived short-chain fatty acids can produce ASD-like behavior and alter brain lipid composition. In this paper, we report that repeated brief intraventricular infusion of both PPA and BUT induce ASD-like behavioral changes in adult rats, which were accompanied by alterations in brain phospholipid and acylcarnitine profiles consistent with finding in the blood of ASD patients. This study provides further evidence for the validity of the PPA rodent model of autism and suggests this model may prove to be a useful tool in understanding the relationship between abnormal lipid metabolism and the complex pathogenesis of ASD.

Materials and methods

Subjects

Seventeen naive male Long-Evans rats (Charles River Laboratories, Quebec, Canada), weighing 200–225 g (approximately 47–49 days old) at the start of the experiment, were individually housed at $21 \pm 1^\circ\text{C}$ in standard acrylic cages ($26 \times 48 \times 21$ cm) and exposed to 12 : 12 h light–dark cycle (lights on 07.00 to 19.00 hours). Animals were allowed access to food (Prolab rat chow) and tap water *ad libitum*. All procedures were in accordance with guidelines of the Canadian Council on Animal Care and were approved by the University of Western Ontario Animal Use Subcommittee.

Surgical implantation of intracerebroventricular cannula

To induce anesthesia, animals were placed in a sealed Plexiglas box into which 4% isoflurane and 2 L/min oxygen flow were introduced. The animal was then placed into a Kopf stereotaxic frame equipped

with a gas flow nose cover to maintain anesthesia throughout surgery at 2% isoflurane and 500 mL/min oxygen flow. Under aseptic conditions, the animal received an implantation of a 23-gauge guide cannula with the tip in the right lateral ventricle (AP 1.3 mm, ML 1.8 mm, DV 3.0 mm) (Paxinos and Watson 1986). Cannula placements were in accordance with Paxinos and Watson (1986) rat brain atlas and our previous experience (MacFabe *et al.* 2007, 2008; Shultz *et al.* 2008, 2009). A removable obturator sealed the guide cannula until an injection was to be made. To facilitate infusion into the lateral ventricle, the tip of a 30 ga injection cannula protruded 0.5 mm beyond the tip of the guide cannula. 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. Immediately post-surgery, all rats received a subcutaneous injection of analgesic (Ketoprofen, 1 mL/kg). All animals were allowed 14 days recovery before behavioral testing took place.

Treatment groups and intracerebroventricular infusion procedure

Following recovery, animals were assigned to one of three groups: PPA treatment (4.0 μL of a 0.26 M solution, $n = 6$); BUT (4.0 μL isomolar solution to PPA, $n = 5$); phosphate-buffered saline (PBS) control (4.0 μL , $n = 6$). PPA and BUT was dissolved in PBS vehicle and buffered to pH 7.5 using concentrated HCl or NaOH. Each group received intracerebroventricular (ICV) infusions twice daily, separated by 4 h, for seven consecutive days. Compounds were infused using a 30 ga injection cannula connected to a Sage syringe pump with sterile PE10 tubing. The tip of the injection cannula protruded 0.5 mm beyond the tip of the guide cannula. Each injection consisted of 4.0 μL of solution delivered over a period of 1 min. The infusion cannula remained in place for an additional minute before being removed.

Apparatus: automated activity monitors

Locomotor activity was monitored using eight Versamax Animal Activity Monitors (AccuScan Model DCM-8, Columbus, OH, USA). Each monitor consisted of a plexiglas open field chamber ($40 \times 40 \times 30.5$ cm), and a Plexiglas lid with air holes. Located on all four sides of the chamber were 16 infrared beam sensors 2.54 cm apart and 4.5 cm from the floor to measure horizontal movements and on two opposite two sides were 16 infrared beams located 15 cm above the chamber floor to measure the vertical movements. Light levels at the floor of each open-field were approximately 900 lux. A VersaMax Analyser (AccuScan Model VSA-16) processed relayed data from each automated open-field to a computer located in an adjacent room to the testing chambers.

Behavioral testing

Animals were habituated to the activity monitors for two 30-min sessions prior to the treatment sessions. Baseline was recorded on the third day to establish normal activity levels for untreated rats. Locomotor activity assessments were made for 30 min immediately following the second ICV infusion daily for 7 days. Locomotor assessments were in terms of horizontal and vertical activity measures, and repetitive movement (stereotypy) with the current work presenting total distance traveled in (cm) and number of stereotypic movements. Twenty-four hours after animals received the final ICV infusion, animals were decapitated, brains quickly

removed, frozen on dry ice and stored in a -70°C freezer prior to lipid analysis.

Lipid extraction

Brain tissue (100 mg) ipsilateral to ICV infusion cannula, containing frontal cortex, striatum, thalamus and dorsal hippocampus, 3.0 mm posterior to bregma (Paxinos and Watson 1986) was ground to fine powder while frozen in liquid nitrogen. The ground sample (100 mg) was extracted with a mixture (2.5 mL) of chloroform/methanol/0.01% butylated hydroxytoluene (2 : 1 : 0.0003; v/v/wt) in centrifuge tubes. The sample and solvent mixture was centrifuge at 10 000 *g* for 15 min. After centrifugation, the supernatant was collected and 100 μL of 0.4 mg/mL octadecane added as internal standard. Following the addition of the internal standard, 1 mL of 0.25% potassium chloride (KCl) was added and the sample incubated in a water bath at 70°C for 10 min. After incubation, the sample was removed, cooled and the aqueous layer (top) removed with a Pasteur pipette. The remaining layer (organic) containing the lipids was transferred to a new pre-weighed 4 mL sample vial having a poly-teflon resin-lined cap (VWR). The sample was dried under a gentle stream of nitrogen and the vial re-weighed to determine the amount of sample recovered. The recovered sample was then resuspended in 1 mL chloroform : methanol (2 : 1 v/v) and stored at -20°C for further analysis (Sherma and Fried 2003).

Preparative TLC

TLC was used to separate the individual phospholipids present in the sample. Ten microliter aliquots were taken from the sample stored at -20°C and spotted on 20 cm \times 20 cm glass plates coated with silica gel (K6F: 250 μM layer thickness, pore size 60 \AA) (Fisher Scientific, Pittsburgh, PA, USA) using a 10- μL Hamilton syringe. A sharp pencil was used to mark lanes on the plate before samples were spotted. Reference standards [cardiolipin (CL), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylcholine (PC), phosphatidylinositol (PI) and phosphatidylethanolamine (PE)] obtained from Sigma (St Louis, MO, USA), were spotted separately in the first set of lanes and brain samples spotted in subsequent lanes. TLC plates containing the reference standards and brain samples were placed in TLC chambers containing a solvent system of chloroform: methanol : acetic acid: water (50 : 37.5 : 3.5 : 2 v/v/v/v) and developed to separate the lipid components (Sherma and Fried 2003) Following development, the plates were dried using a hair dryer, and placed in another TLC chamber containing iodine crystals. Separated lipid components were visible as bright yellow or brown spots on the plates. The plates were removed from the chamber and the spots circled with a pencil. The reference standards were then used to identify the separated lipid components in the samples. The separated lipid components were scraped from the plates and placed in centrifuge tubes containing 1 mL chloroform : methanol (2 : 1 v/v) and 0.1 mg/mL triacontane added as internal standard. The tubes were centrifuged at 10 000 *g* for 15 min and the supernatant collected in 2 mL sample vials with poly-teflon resin lined caps and dried under a gentle stream of nitrogen. The dried sample was derivatized for gas chromatography (GC) analysis.

GC analysis

GC analysis was conducted according to the combined methods of (Chow *et al.* 1989) and (Bicalho *et al.* 2008). Briefly, 200 μL of

0.5 N methanolic-HCl (Sigma) was added to the dried sample and the sample heated in a drying oven at 90°C for 30 min. The sample was then removed from the oven and cooled in a fume hood. Distilled water (0.8 mL) was added to the sample mixture and the mixture extracted thrice using 500 μL of hexane each time. The hexane fractions were pooled (1500 μL), dried under nitrogen, and the residue re-suspended in 50 μL of hexane containing butylated hydroxytoluene (1 $\mu\text{g}/\text{mL}$). Plasmalogens in the total lipid extract were converted to dimethyl acetals (DMA) and fatty acids converted to fatty acid methyl esters (FAME). Quantitative analysis of the DMA and FAME present in the samples were carried out on an Agilent 6890N gas chromatograph coupled with a flame ionization detector (GC-FID) (Agilent Technologies, Santa Clara, CA, USA) using a DB-23 high resolution column (30 m \times 250 μm ID \times 0.25 μm film thickness). Qualitative analysis was performed simultaneously using a GC/MS machine (GC: Varian 3500, MS: Finnigan MAT 8400) also fitted with a DB-23 high resolution column. With both machines, the samples (1 μL) were injected in splitless mode according to the following temperature profile: injection at 80°C (held for 2 min), temperature ramped to 180°C at 5°C per min, then to 200°C at 1.5°C per min, then to 240°C at 10°C per min and held for 3 min. The peaks were identified through mass spectrum elucidation from literature references (Bicalho *et al.* 2008; Hartig 2008), comparison of retention times and mass spectra obtained from commercial standards (Supelco 37 component mix, Supelco PUFA No. 3, Supelco FAME mix C8-C24, C16 DMA and C10 DMA, Sigma). The amounts of individual fatty acids identified were calculated using standard curves prepared from the standard mixtures and values expressed on a percent by weight basis.

Carnitine and acylcarnitine analysis

Carnitine and acylcarnitine analysis was conducted according to the combined methods of Aureli *et al.* (2000) and Cyr *et al.* (2000). Briefly, 100 mg of brain sample frozen in liquid nitrogen was ground to powder in a steel mortar. The ground samples were added to flash frozen centrifuge tubes and 1 mL chloroform containing 50 mg/L of butylated hydroxytoluene was added. Milli-Q water (1 mL) and 50 μL labeled internal standards (NSK-B; Cambridge Isotope Laboratories, Andover, MA, USA) were added and the samples centrifuged at 10 000 *g* for 15 min. Following centrifugation, the chloroform layer was removed and discarded. The remaining layer (aqueous) was acidified to pH 2 using 30 μL of 1 N HCl and the acylcarnitines extracted twice using 1 mL butanol each time. The butanol fractions were pooled (2 mL) and anhydrous sodium sulfate added to remove water. A Pasteur pipette was used to transfer the butanol into new pre-weighed sample vials and the samples dried under nitrogen. The vials were re-weighed to determine the amount of sample recovered after extraction. The recovered samples were re-suspended in 1 mL of butanol and aliquots (150 μL per sample) were used to derivatize carnitine and acylcarnitine to butyl esters and the remaining sample stored at -20°C for further analysis.

Derivatization of carnitine and acylcarnitine to butyl esters

Aliquots (150 μL) of the extracted samples were dried under nitrogen and 500 μL of 3 N butanolic-HCl was added. The sample was heated at 65°C for 15 min. After butylation, the samples were

dried under nitrogen and re-suspended in 100 μ L of acetonitrile : water (4 : 1 v/v) and analyzed using electrospray ionization mass spectrometry (Morris *et al.* 2005).

Electrospray ionization mass spectrometry

Carnitine samples were analyzed by infusion on a triple quadruple instrument (Micromass QuattroMicro; Waters, Milford, MA, USA). The mass spectrometer was operated in the positive ion mode and acylcarnitine butyl esters detected using a precursor ion of m/z 85. The investigated mass range was from 100 to 700 m/z . The cone voltage was set at 30 V, the collision energy at 25–40 V, source temperature at 80°C and the collision gas at 2e-3. Quantitative analysis was achieved using MassLynx 4.0 (Micromass) software by comparing the signal intensity of an analyte against the corresponding deuterium labeled internal standard. Data are expressed as nmol/mg brain tissue.

Statistical analysis

A total of 17 animals were used for this study (6 PBS, 5 BUT and 6 PPA). General analysis of variance (ANOVA) was used to determine the effects of treatments on lipid components. Where treatment effects were significant, the means were compared with Fisher's LSD test, $\alpha = 0.05$. The data were analyzed using the Statistix software package (Analytical Software, Tallahassee, FL, USA).

Results

Assessment of animal behavior

Locomotor behavior was evaluated daily for 7 days immediately after the second infusion of the day with PBS, PPA and BUT. Assessment of baseline behavior, prior to drug treatment, yielded no significant difference between groups of animals. Locomotor activity was expressed as the total distance traveled by each animal and the number of stereotypic/repetitive movements displayed by each animal (Fig. 1a and b). The results revealed that total distance traveled ($p < 0.01$) and number of stereotypic movements ($p < 0.05$) were significantly higher in animals infused with PPA and BUT compared with the controls (PBS) (Fig. 1). The total distance traveled was significantly higher in PPA-treated animals on infusion day 2 compared with both BUT and PBS animals. On infusion days 3, 4 and 7, both PPA- and BUT-treated animals traveled greater distances than PBS-treated animals; however, there was no significant difference between PPA and BUT on these days. On infusion day 6, only the BUT-treated animals traveled significantly greater distances than the PBS-treated animals (Fig. 1a). A similar trend was observed in stereotypy, where on infusion days 2, 3, 4, 6 and 7 both PPA- and BUT-treated animals displayed greater stereotypic movements compared with the control animals. Only on infusion day 1 did PPA-treated animals perform less stereotypic movements compared with the control animals (Fig. 1b). Animals were killed 24 h following the last infusion and behavioral assessment. Brain lipids were evaluated to determine if there was any evidence

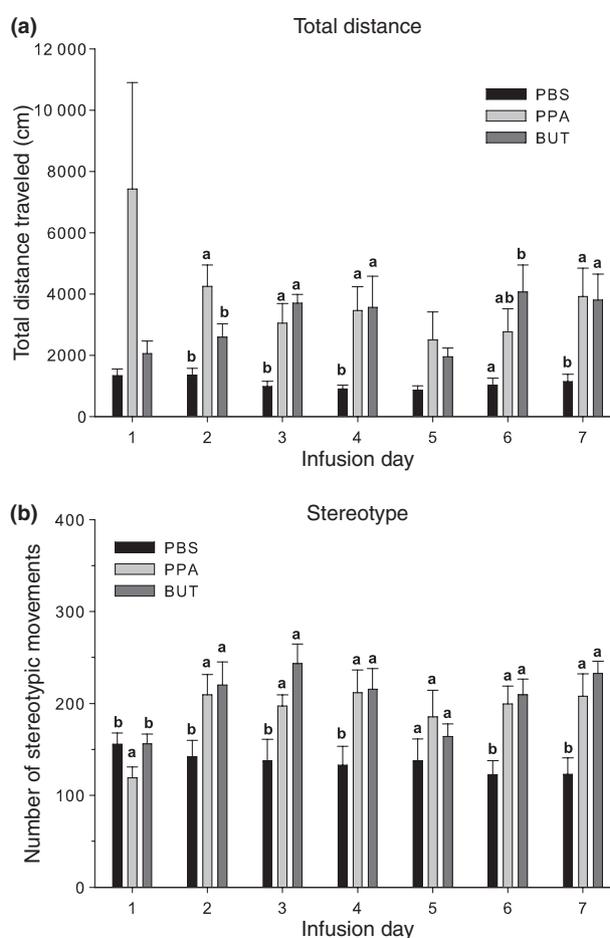


Fig. 1 Total distance traveled (a) and number of stereotypic movements (b) representing abnormal animal behavior following infusions with PPA and BUT. Animals received ICV infusions of PPA ($n = 6$), BUT ($n = 5$), or PBS ($n = 6$) twice a day for seven consecutive days. Locomotor activity and horizontal movements were measured immediately after each infusion for 30 min. Values represent mean \pm SE. Mean values accompanied by different letters (e.g. a, b) indicate significant difference between treatments at, LSD = 0.05, $n = 6$ per experimental replicate. PBS, phosphate-buffered saline solution; BUT, butyric acid; PPA, propionic acid.

for alterations in lipid composition associated with ICV infusions with PPA and BUT.

Phospholipid analysis

Brain phospholipids were separated into SM, CL, PS + PI, PC, and PE following preparative TLC analysis. Hydrolysis of these separated phospholipid components followed by GC analysis revealed significant quantitative changes in fatty acid components following ICV infusion with PPA or BUT compared with the PBS-treated animals (Fig. 2a–e). There was a consistent increase in total saturated fatty acids in all of the separated phospholipid components following infusion

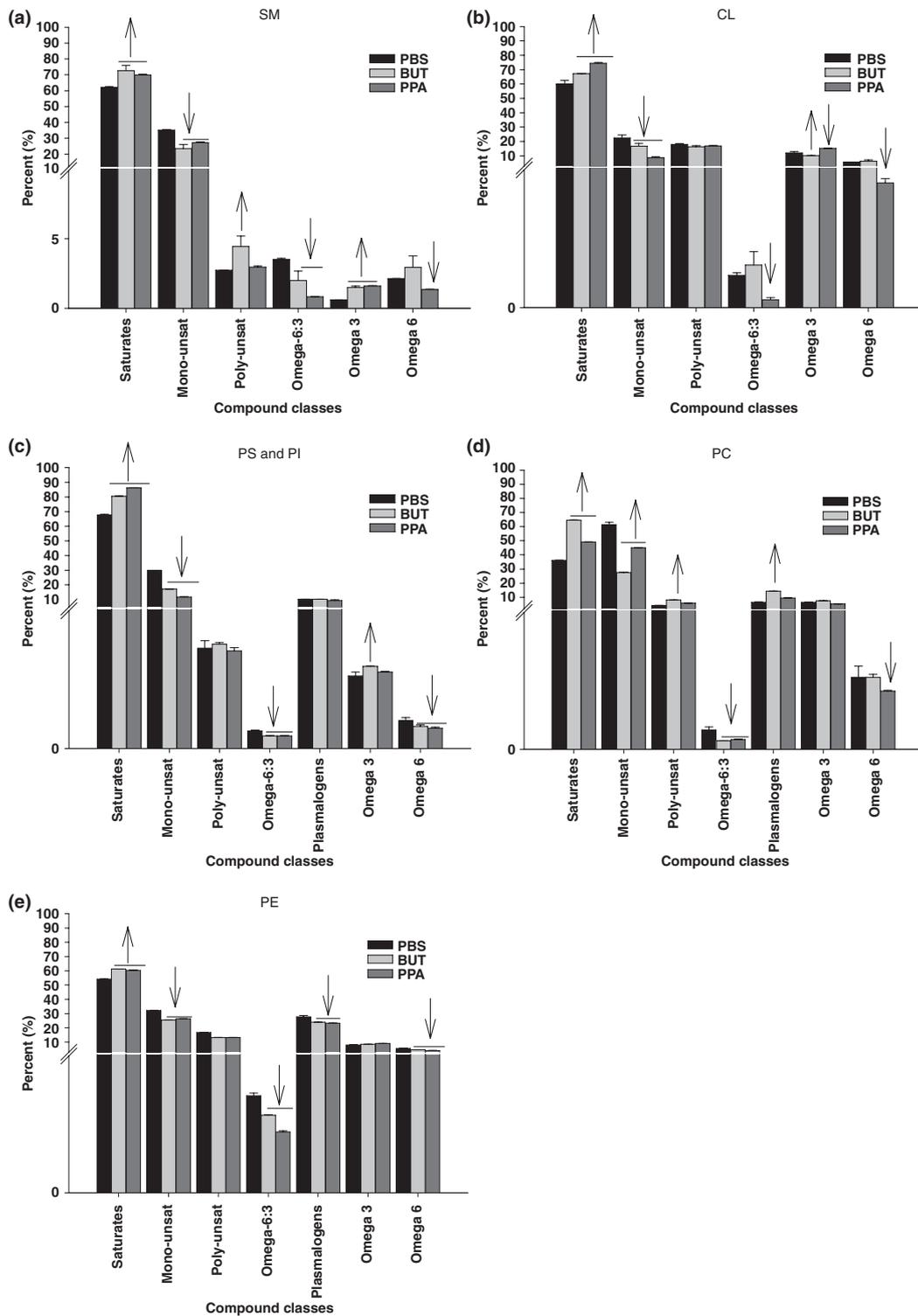


Fig. 2 Changes in (a) spingomyelin, (b) cardiolipin, (c) phosphatidylserine and inositol, (d) phosphatidylcholine and (e) phosphatidylethanolamine in rat brain tissue following intracerebroventricular infusion with phosphate-buffered saline (PBS), butyric acid (BUT) and propionic acid (PPA). Values represent mean \pm SE. Arrows indicate

significant difference between treatments [PPA and BUT compared with the control (PBS)]; and direction of the changes (increase or decrease) at LSD = 0.05, $n = 6$ per treatment. Monounsaturates; polyunsaturates.

with BUT and PPA compared with the control, with PPA-treated animals having higher amounts than BUT-treated animals in the CL (Fig. 2b) and PS + PI (Fig. 2c) fractions. In the PC fraction (Fig. 2d), the BUT-treated animals had higher amounts of saturates than both PPA- and PBS-treated animals. Evaluation of the fatty acid composition of each phospholipid component revealed that significant ($p < 0.001$) increases in C16:0 and C18:0 fatty acids accounted for the observed increases in total saturated fatty acids in all of the phospholipid components following infusion with either PPA or BUT (Tables 1–5). Conversely, there was a consistent

Table 1 Fatty acid composition (percent by weight) of sphingomyelin in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
C14:0	2.85 ± 0.01 ^b	3.41 ± 0.17 ^a	2.71 ± 0.03 ^b
C16:0	21.86 ± 0.31 ^a	26.49 ± 0.30 ^b	25.53 ± 1.30 ^b
C16:1	1.18 ± 0.11 ^a	3.57 ± 0.63 ^b	2.97 ± 0.46 ^b
C18:0	36.10 ± 0.59 ^a	39.79 ± 0.32 ^b	39.76 ± 0.64 ^b
C18:1n9	28.98 ± 0.19 ^a	16.08 ± 2.88 ^b	16.03 ± 0.71 ^b
C18:1n7	4.97 ± 0.13 ^a	3.75 ± 0.92 ^a	8.14 ± 1.53 ^b
C18:2n6	2.12 ± 0.03 ^a	2.93 ± 0.84 ^a	1.35 ± 0.05 ^b
C18:3n3	0.60 ± 0.02 ^a	1.53 ± 0.09 ^b	1.60 ± 0.03 ^b
C22:0	1.34 ± 0.02 ^a	2.45 ± 0.55 ^b	1.91 ± 0.15 ^a
Total	100	100	100

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. C represents fatty acids as fatty acid methyl esters.

Table 2 Fatty acid composition (percent by weight) of cardiolipin in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
C14:0	2.84 ± 0.12 ^a	1.60 ± 0.07 ^b	2.66 ± 0.06 ^a
C16:0	29.06 ± 0.83 ^a	23.29 ± 0.05 ^b	34.16 ± 0.33 ^c
C16:1	3.19 ± 0.13 ^a	2.71 ± 1.96 ^b	2.52 ± 0.79 ^c
C18:0	27.79 ± 2.30 ^a	42.20 ± 0.54 ^b	37.52 ± 0.24 ^c
C18:1n9	16.81 ± 2.29 ^a	12.31 ± 0.37 ^b	4.57 ± 0.02 ^c
C18:1n7	2.47 ± 0.10 ^a	1.57 ± 0.43 ^b	1.65 ± 0.04 ^b
C18:2n6	3.24 ± 0.15 ^a	4.43 ± 1.91 ^a	1.26 ± 0.01 ^b
C18:3n3	3.25 ± 0.23 ^a	1.64 ± 0.01 ^b	7.77 ± 0.09 ^c
C20:4n6	2.47 ± 0.10 ^a	1.79 ± 0.01 ^b	0.54 ± 0.05 ^c
C22:6n3	8.88 ± 0.62 ^a	8.46 ± 0.04 ^a	7.34 ± 0.04 ^b
Total	100	100	100

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. C represents fatty acids as fatty acid methyl esters.

Table 3 Fatty acid composition (percent by weight) of phosphatidylserine and inositol in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
P16:1	9.85 ± 0.03 ^a	9.85 ± 0.05 ^a	8.79 ± 0.22 ^b
C16:0	44.45 ± 0.19 ^a	50.63 ± 0.03 ^b	53.64 ± 0.50 ^c
C16:1	0.50 ± 0.06	0.51 ± 0.01	0.38 ± 0.10
P18:0	0.35 ± 0.01	0.38 ± 0.01	0.63 ± 0.26
C18:0	22.71 ± 0.10 ^a	29.04 ± 0.06 ^b	31.48 ± 0.14 ^c
C18:1n9	19.34 ± 0.06 ^a	6.01 ± 0.04 ^b	2.09 ± 0.02 ^c
C18:1n7	0.17 ± 0.02 ^a	0.80 ± 0.01 ^b	0.39 ± 0.05 ^c
C18:2n6	0.61 ± 0.07 ^a	0.48 ± 0.03 ^b	0.45 ± 0.02 ^b
C18:3n3	0.29 ± 0.01	0.28 ± 0.01	0.30 ± 0.01
C20:0	0.16 ± 0.13	0.23 ± 0.01	0.15 ± 0.06
C22:0	0.33 ± 0.02	0.29 ± 0.01	0.35 ± 0.01
C22:6n3	1.28 ± 0.09 ^a	1.50 ± 0.01 ^b	1.35 ± 0.01 ^a
Total	100	100	100

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. C represents fatty acids as fatty acid methyl esters and P represents plasmalogens as dimethyl acetals.

Table 4 Fatty acid composition (percent by weight) of phosphatidylcholine in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
C14:0	0.35 ± 0.01 ^a	0.57 ± 0.08 ^b	0.47 ± 0.01 ^b
P16:0	0.25 ± 0.01	0.33 ± 0.03	0.24 ± 0.03
P16:1	5.49 ± 0.02 ^a	12.97 ± 0.03 ^b	8.55 ± 0.01 ^c
C16:0	22.61 ± 0.07 ^a	44.00 ± 0.03 ^b	33.88 ± 0.25 ^c
C16:1	0.21 ± 0.01 ^a	0.31 ± 0.02 ^b	0.45 ± 0.05 ^c
P18:0	0.45 ± 0.04	0.52 ± 0.02	0.50 ± 0.10
P18:1	0.25 ± 0.23	0.39 ± 0.01	0.31 ± 0.02
C18:0	12.12 ± 0.01 ^a	18.35 ± 0.02 ^b	13.47 ± 0.03 ^c
C18:1n9	53.35 ± 0.07 ^a	13.07 ± 0.09 ^b	35.49 ± 0.17 ^c
C18:1n7	0.15 ± 0.01	0.14 ± 0.01	0.16 ± 0.01
C18:2n6	0.44 ± 0.07 ^a	0.32 ± 0.04 ^{ab}	0.29 ± 0.01 ^b
C18:3n3	0.13 ± 0.02 ^a	0.23 ± 0.01 ^b	0.14 ± 0.01 ^a
C20:0	0.14 ± 0.01	0.24 ± 0.05	0.34 ± 0.25
C20:1n9	0.16 ± 0.01	0.59 ± 0.47	0.10 ± 0.01
C20:4n6	0.09 ± 0.01 ^a	0.21 ± 0.01 ^b	0.13 ± 0.01 ^a
C22:0	0.28 ± 0.01 ^a	0.48 ± 0.08 ^b	0.29 ± 0.02 ^a
C22:6n3	3.53 ± 0.01 ^a	7.28 ± 0.06 ^b	5.19 ± 0.04 ^c
Total	100	100	100

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. C represents fatty acids as fatty acid methyl esters and P represents plasmalogens as dimethyl acetals.

Table 5 Fatty acid composition (percent by weight) of phosphatidylethanolamine in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
C14:0	0.61 ± 0.65	0.30 ± 0.01	0.21 ± 0.01
P16:0	3.88 ± 0.01 ^a	3.87 ± 0.01 ^a	2.55 ± 0.13 ^b
P16:1	14.37 ± 0.04 ^a	14.31 ± 0.10 ^a	15.77 ± 0.39 ^b
C16:0	10.76 ± 0.10 ^a	12.14 ± 0.05 ^b	12.61 ± 0.18 ^b
C16:1	4.46 ± 0.13 ^a	3.14 ± 0.05 ^b	1.96 ± 0.16 ^c
P18:0	4.57 ± 0.44 ^a	2.45 ± 0.10 ^b	2.00 ± 0.01 ^c
P18:1	4.95 ± 0.38 ^a	3.37 ± 0.10 ^b	2.97 ± 0.01 ^b
C18:0	32.31 ± 0.65 ^a	41.53 ± 0.03 ^b	42.67 ± 0.21 ^b
C18:1n9	5.10 ± 0.14 ^a	3.78 ± 0.02 ^b	3.81 ± 0.19 ^b
C18:1n7	2.36 ± 0.04 ^a	0.56 ± 0.01 ^b	0.41 ± 0.03 ^b
C18:2n6	1.73 ± 0.02 ^a	0.43 ± 0.02 ^b	0.64 ± 0.01 ^b
C18:3n3	3.96 ± 0.17 ^a	2.82 ± 0.01 ^b	4.01 ± 0.31 ^a
C20:0	0.71 ± 0.01	0.37 ± 0.01	0.40 ± 0.02
C20:1	0.97 ± 0.07 ^a	0.36 ± 0.01 ^b	1.45 ± 0.08 ^a
C20:4n6	3.93 ± 0.01 ^a	4.36 ± 0.01 ^b	3.44 ± 0.13 ^a
C22:0	1.26 ± 0.06 ^a	0.54 ± 0.19 ^b	0.01 ± 0.01 ^c
C22:6n3	4.07 ± 0.07 ^a	5.67 ± 0.06 ^b	5.09 ± 0.42 ^b
Total	100	100	100

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. C represents fatty acids as fatty acid methyl esters and P represents plasmalogens as dimethyl acetals.

decline ($p < 0.001$) in total monounsaturated fatty acids in all of the measured phospholipid components following infusions with BUT and PPA (Fig. 2a–e), with the greatest decline in total monounsaturates occurring in the CL (Fig. 2b) and PS + PI (Fig. 2c) fractions of PPA-treated animals, and the PC fraction of BUT-treated animals (Fig. 2d). Analysis of the fatty acid composition of each phospholipid component showed that C18:1n9 fatty acid was the major contributor to the decline in total monounsaturates following infusion with either PPA or BUT (Tables 1–5). Changes in plasmalogens, $\omega 6 : \omega 3$ ratios, $\omega 3$, $\omega 6$ and polyunsaturated fatty acids were more varied following treatments with PPA and BUT (Fig. 2a–e). Plasmalogens were observed in the PS + PI (Fig. 2c), PC (Fig. 2d), and PE (Fig. 2e) fractions of the phospholipids and their quantities increased in the PC fraction of BUT-treated animals but declined in the PE fractions of both PPA- and BUT-treated animals. Conversion of the vinyl ethers present in the plasmalogen forms of these phospholipids to dimethyl acetals was used to estimate the amount of specific plasmalogen components present in the brain tissue. Analysis of the plasmalogen composition showed that C16:1 vinyl ether increased in the PC fraction of both PPA and BUT treated animals and was responsible for the difference observed in total plasmalogen accumulation (Table 4). In

the PE fraction, a decline in C18:0 and C18:1 vinyl ethers in BUT-treated animals as well as a decline in C16:0 vinyl ether in PPA-treated animals accounted for the decline in total PE plasmalogens observed in both PPA- and BUT-treated animals (Table 5).

Total polyunsaturated fatty acid increased in SM (Fig. 2a) and PC (Fig. 2d) fractions of BUT-treated animals, but was unchanged in the corresponding fractions of PPA-treated animals. In BUT-treated animals, increases in C18:2n6 and C18:3n3 fatty acids accounted for the increase in total SM polyunsaturates (Table 1), whereas C22:6n3 accounted for the increase in total polyunsaturates in the PC fraction (Table 4). There was a consistent decline in total $\omega 6$ fatty acids in all the phospholipid fractions of PPA-treated animals (Fig. 2a–e), whereas in BUT-treated animals this decline was observed only in the PS + PI (Fig. 2c), and PE fractions (Fig. 2e). A decline in C18:2n6 fatty acid accounted for this difference observed in total $\omega 6$ fatty acid accumulation (Tables 1–5), except in the CL fractions of PPA-treated animals, where both C20:4n6 and C18:2n6 fatty acids were responsible for the decline in total $\omega 6$ accumulation (Table 2). Total $\omega 3$ fatty acids on the other hand, increased in SM (Fig. 2a) and CL (Fig. 2b) following infusions with PPA; but decreased in CL, and increased in SM (Fig. 2a) and PS + PI (Fig. 2c) following infusions with BUT. Alterations in C18:3n3 fatty acids in SM (Table 1) and CL (Table 2), and alterations of C22:6n3 fatty acids in PS + PI (Table 3) accounted for the differences observed in total $\omega 3$ fatty acids of these phospholipid components following infusions with PPA and BUT. The changes observed in total $\omega 3$ and $\omega 6$ fatty acids accumulation following infusion with PPA and BUT resulted in significant alterations ($p = 0.001$) in the $\omega 6 : \omega 3$ fatty acid ratio (Fig. 2a–e). There was a consistent decrease in the ratio of $\omega 6 : \omega 3$ fatty acids in all of the phospholipid components following infusion with PPA. This trend was also observed after infusion with BUT, except in CL where there was an increase in the ratio of $\omega 6 : \omega 3$ fatty acids.

Carnitine and acylcarnitine analysis

Carnitine and acylcarnitines were analyzed as butyl ester derivatives. The results showed a trend toward lower free carnitine in BUT- and PPA-treated animals; however, this trend was not significant ($p = 0.90$) when compared with control animals (Fig. 3a). Conversely, there was a consistent significant ($p = 0.02$) increase in total acylcarnitines (Fig. 3b), total long-chain (C12–C24) acylcarnitines (Fig. 3c), total short-chain (C2–C9) acylcarnitines (Fig. 3d) and the ratio of bound to free carnitine following infusions with PPA and BUT.

Increases in the accumulation of the following acylcarnitines: C2:0, C14:1, C15:0, C16:0, C16:1, C18:0, C18:1, C22:0, C22:1 and C24:0 accounted for the increase levels of total acylcarnitines observed following infusions with PPA

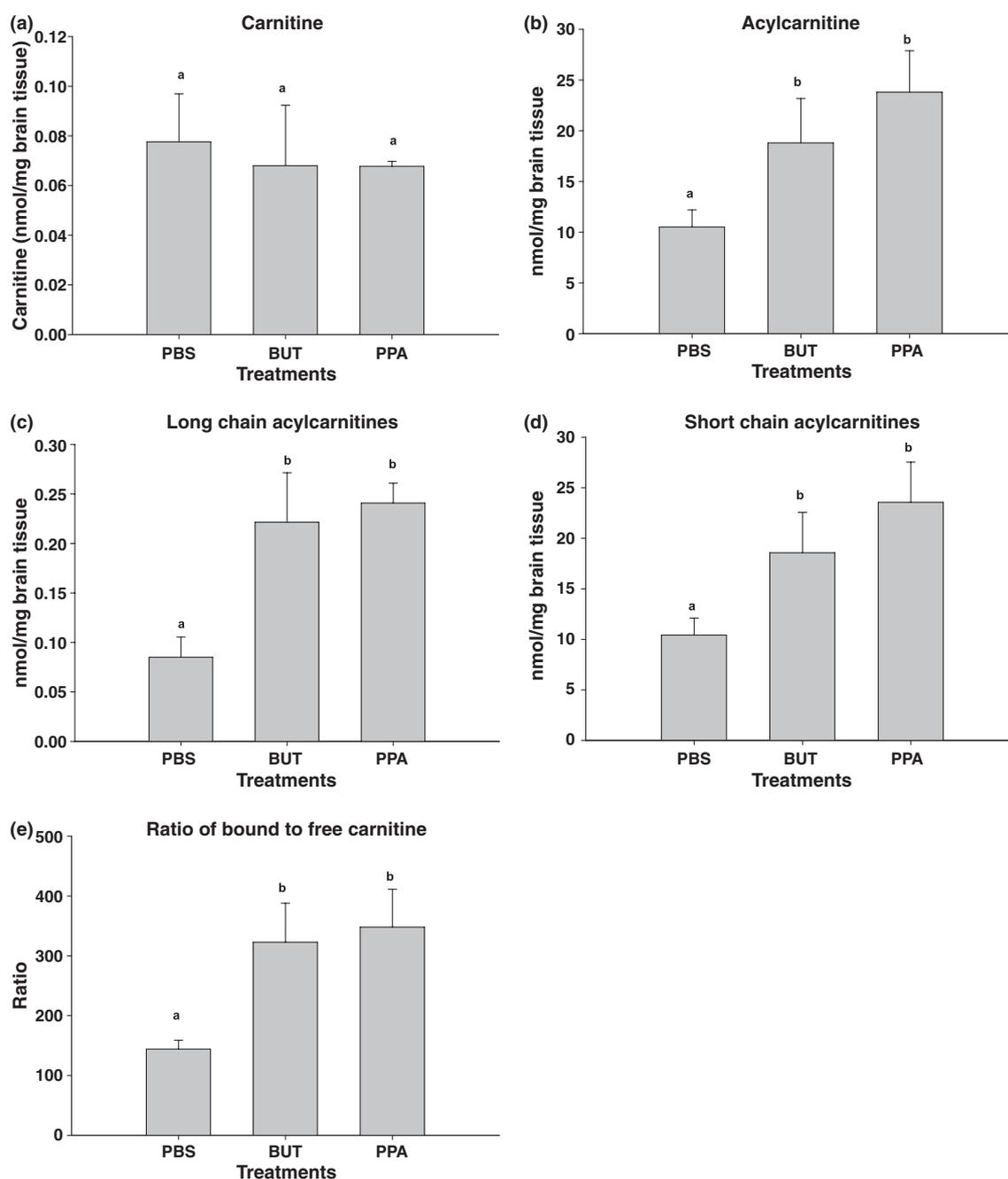


Fig. 3 Changes in (a) carnitine, (b) acylcarnitine, (c) long-chain acylcarnitine, (d) short-chain acylcarnitine and (e) ratio of bound to free carnitine in rat brain tissue following intracerebroventricular infusion with phosphate-buffered saline (PBS), butyric acid (BUT) and propionic acid

(PPA). Values represent mean \pm SE. Mean values accompanied by different superscript letters (e.g. a, b and c) indicate significant difference between treatments at LSD = 0.05, $n = 6$ per treatment. Long-chain acylcarnitine = C12 to C24; short-chain acylcarnitine = C2 to C9.

and BUT. When these acylcarnitines were grouped according to chain length, acetyl carnitine (C2) was the major contributor to the increases observed in short-chain acylcarnitines, whereas C16:0, C18:0, C18:1, C22:0 and C22:1 were the major contributors to the increases observed in long-chain acylcarnitines (Table 6).

Discussion

Behavior

In this study, both PPA- and BUT-treated rats exhibited greater locomotor activity, as measured by number of stereotypic movements and total distance traveled. These

Table 6 Changes in carnitine and acylcarnitine levels (nmol/mg) in rat brains after treatment with propionic acid (PPA), butyric acid (BUT) and phosphate-buffered solution (PBS)

Composition	PBS	BUT	PPA
Carnitine	0.08 ± 0.02	0.07 ± 0.02	0.07 ± 0.002
C2	10.40 ± 1.67 ^a	18.54 ± 4.00 ^b	23.52 ± 4.00 ^b
C3	0.003 ± 0.001	0.005 ± 0.003	0.004 ± 0.001
C4	0.008 ± 0.001	0.01 ± 0.005	0.01 ± 0.005
C5	0.002 ± 0.001	0.003 ± 0.001	0.008 ± 0.002
C6	0.002 ± 0.001	0.002 ± 0.001	0.003 ± 0.001
C8	0.003 ± 0.001	0.004 ± 0.002	0.003 ± 0.001
C9	0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001
C12	0.003 ± 0.001	0.005 ± 0.002	0.05 ± 0.003
C14	0.006 ± 0.003	0.006 ± 0.002	0.01 ± 0.008
C14:1	0.001 ± 0.001 ^a	0.002 ± 0.001 ^a	0.008 ± 0.004 ^b
C14:2	0.002 ± 0.001	0.003 ± 0.002	0.002 ± 0.001
C15	0.002 ± 0.001 ^a	0.004 ± 0.002 ^a	0.008 ± 0.005 ^c
C16	0.008 ± 0.003 ^a	0.04 ± 0.02 ^b	0.03 ± 0.006 ^b
C16:1	0.002 ± 0.001 ^a	0.006 ± 0.002 ^b	0.006 ± 0.002 ^b
C17	0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001
C18	0.007 ± 0.001 ^a	0.01 ± 0.002 ^b	0.01 ± 0.005 ^b
C18:1	0.01 ± 0.005 ^a	0.03 ± 0.008 ^b	0.03 ± 0.007 ^b
C20	0.001 ± 0.001	0.002 ± 0.001	0.003 ± 0.001
C21	0.004 ± 0.002	0.002 ± 0.001	0.001 ± 0.001
C22	0.02 ± 0.01 ^a	0.04 ± 0.01 ^b	0.04 ± 0.007 ^b
C22:1	0.02 ± 0.004 ^a	0.07 ± 0.02 ^b	0.08 ± 0.01 ^b
C24	0.004 ± 0.001 ^a	0.01 ± 0.005 ^b	0.007 ± 0.001 ^c

Values (percent by weight) represent mean ± standard errors. Mean values in the same row accompanied by different superscript letters (e.g. a, b and c) are significantly different between treatments at LSD = 0.05, $n = 9$. Numbers following C represents carbon number of the fatty acid moiety attached to carnitine. Short-chain represents C2–9 acylcarnitines and long-chain represents C12–24 acylcarnitines.

findings are consistent with previous studies via automated analysis and direct visual observations which revealed that PPA-treated rats showed elevated locomotor activity, including increased turning behaviors, snake-like postures, limb dystonia, and retropulsion. (MacFabe *et al.* 2007, 2008). These behaviors bear some resemblance to the hyperactive and repetitive behaviors which are core symptoms of ASD (Volkmar *et al.* 2004). In this study, we report that like PPA, isomolar intraventricular infusions of BUT, produce similar increases in locomotor activity. Interestingly, these increases in locomotor activity were not observed when propanol, the non-acidic (alcohol) analog to PPA was used (MacFabe *et al.* 2007; Parab *et al.* 2007). Intracellular neuronal acidification produces widespread effects on neurotransmitter release including glutamate, dopamine, norepinephrine, and serotonin, each of which plays a major role in the elicitation of locomotor activity (Smith *et al.* 1997; Remblin *et al.* 1999; Filosa *et al.* 2002; Cannizzaro *et al.* 2003; Severson *et al.* 2003). Therefore, intracellular pH reduction by PPA, and

possibly BUT, provides a possible explanation for the short-chain fatty acid induced increases in locomotor activity observed in the present study. These findings indicate that PPA along with other short-chain fatty acids can elicit behavioral changes reminiscent of ASD in rodents.

Lipid analysis

Following the observation of behavioral changes associated with BUT and PPA infusions, brain lipids were analyzed to determine if there were any alterations in brain lipid composition associated with these behavioral changes. Several reports have presented evidence for altered lipid profiles in the blood of autistic patients compared with control individuals (Bell *et al.* 2000, 2004; Vancassel *et al.* 2001; Pastural *et al.* 2009; Wiest *et al.* 2009). In addition, Hu *et al.* (2006) have recently reported alterations in the expression of lipid and steroid implicated genes in ASD lymphoblasts. Altered lipid profiles were observed in rat brain phospholipids following infusion with both BUT and PPA, and in many cases the changes in brain lipid composition were consistent with those observed in the blood of autistic patients. Although both PPA and BUT resulted in alterations in brain lipid components, the alterations seen in PPA treated animals were more consistent across all of the phospholipid species, and there was a trend toward PPA being more effective in altering brain lipids. Nonetheless it is important to point out that in most cases where there were brain lipid alterations, both PPA and BUT had similar overall effects on the levels of individual fatty acids in corresponding phospholipid compound classes.

Alterations in saturates and monounsaturates

The alterations in fatty acid profiles particularly after PPA infusion resulted in decreased levels of total monounsaturates, total $\omega 6$ fatty acids and elevated levels of total saturates in all of the studied phospholipid species. In addition, a decline in total plasmalogen PE and the ratio of $\omega 6 : \omega 3$ was also present. Elevated levels of saturated fatty acids have been reported in red blood cells (Bell *et al.* 2000, 2004) and plasma (Pastural *et al.* 2009) of several autistic patients. The elevations observed in total brain saturates noted here were accompanied by a concomitant decrease in total monounsaturates, particularly, 18:1n9 fatty acid. A decline in the level of this fatty acid along with several other monounsaturates (20:1n9, 22:1n9, 16:1n7 and PE 24:1n9) was also observed in blood drawn from autistic patients (Bell *et al.* 2004; Bu *et al.* 2006; Wiest *et al.* 2009).

Alterations in polyunsaturates

Some of the most compelling evidence for abnormal lipid metabolism in psychiatric disorders has been based on alterations in the levels of PUFA from patients' blood and brain samples (Edwards *et al.* 1998; Horrobin and Bennett

1999; Bell *et al.* 2004; Meguid *et al.* 2008; Wiest *et al.* 2009). In most instances, these findings point to a general reduction in overall or individual levels of $\omega 3$ and $\omega 6$ fatty acids (18:2n6, 18:3n3, 20:4n6, 20:4n3, 20:5n3, 22:5n6 and 22:6n3) which are essential fatty acids or essential fatty acid derivatives. In autism, the preponderance of evidence also points to a reduction in the levels of blood $\omega 3$ and $\omega 6$ fatty acids (Bell *et al.* 2000, 2004; Vancassel *et al.* 2001; Meguid *et al.* 2008; Wiest *et al.* 2009). Variations in the levels of brain $\omega 3$ and $\omega 6$ fatty acids were observed in our model. There was no change in total polyunsaturates ($\omega 3 + \omega 6$) following infusion with PPA. However, when the polyunsaturates were separated into $\omega 3$ and $\omega 6$, there was a decline in the levels of total $\omega 6$ across all of the phospholipids species, while total $\omega 3$ was either elevated or remained unchanged, resulting in an overall decline in the ratio of $\omega 6 : \omega 3$ fatty acids in the membrane. This is important considering healthy, non-inflammatory eicosanoid balance is maintained throughout the body by way of homeostatic balance between $\omega 3$ and $\omega 6$ fatty acids in cell membranes. There is growing evidence that large imbalance of $\omega 6 : \omega 3$ fatty acids are a heightened risk for pro-inflammatory events that foster neurological diseases (Simopoulos 2006; Kidd 2007). Elevated levels of reactive astrocytes and activated microglia which are known to produce proinflammatory cytokines and nitric oxide were observed in our model following PPA infusion (MacFabe *et al.* 2008). Similar findings were observed in the brains of autistic patients at autopsy (Vargas *et al.* 2004). These findings suggest the possibility that imbalances in the ratios of $\omega 6 : \omega 3$ fatty acids may occur in the pathophysiology of ASD.

Alterations in plasmalogens

Plasmalogens are unique glycerophospholipids present in neuronal membranes. They have an enol ether double bond at the sn-1 position and typically have docosahexanoic (22 : 6) or arachidonic (20 : 4) acid at the sn-2 position of the glycerol backbone. It has been suggested that plasmalogens play a role in cholesterol efflux, membrane fusion, ion transport, act as a reservoir for second messengers, and are antioxidants (Farooqui and Horrocks 2001). Decreased levels of plasmalogens have been reported in several neurological disorders including Alzheimer's (Farooqui and Horrocks 2001) and autism (Bell *et al.* 2000, 2004; Wiest *et al.* 2009). A decline in total plasmalogen PE level was observed in our model following PPA treatment. It has been suggested that plasmalogens protect polyunsaturated fatty acids present in the sn-2 position of the glycerol backbone of other phospholipids from oxidative damage by acting as antioxidants. The decreased level of plasmalogens present in blood in autism patients and in brain in our model may indicate that plasmalogen molecules are consumed during the process of protecting neuronal cells from oxidative damage. Increased

oxidative stress markers have been observed in this model (MacFabe *et al.* 2008) and have been reported as a common feature in autistic patients (Chauhan *et al.* 2004; Chauhan and Chauhan 2006).

Alterations in acylcarnitines

Additional evidence for abnormal lipid metabolism in this model was observed as alterations in acylcarnitine species. A relative decrease in free carnitine, albeit insignificant, was observed. This deficiency was accompanied by a significant increase in the accumulation of long- and short-chain acylcarnitines. Carnitine is essential for transport of fatty acids across the inner mitochondrial membrane for β -oxidation and energy production. Abnormalities in acylcarnitine profile and mitochondrial dysfunction have been observed in autism (Clarke and Clark-Taylor 2004). These authors observed elevations in the levels of some long-chain acylcarnitine species (14 : 1 and 14 : 2). Elevations in 14 : 1 and in other long-chain acylcarnitines (16 : 0, 18 : 0, 18 : 1, 22 : 0 and 22 : 1) were observed in our model. PPA is thought to affect mitochondrial fatty acid metabolism by binding to propionyl Coenzyme A and by sequestering carnitine (Roe *et al.* 1984; Brass *et al.* 1986; Wajner *et al.* 2004). Elevation in the levels of these acylcarnitines indicates a potential metabolic disturbance by PPA infusions affecting mitochondrial β -oxidation and bioenergetics.

Cardiolipin alterations and mitochondrial function

Further evidence for alterations in mitochondrial fatty acid metabolism using this model was observed in the levels of CL fatty acid components. CL is a phospholipid found almost exclusively in the inner mitochondrial membrane and is involved in maintaining mitochondrial functionality and membrane integrity (Kiebish *et al.* 2008). PPA infusions increased the levels of saturated and $\omega 3$ fatty acids, while decreasing the levels of monounsaturated and $\omega 6$ fatty acids derived from CL. These alterations are consistent with an impairment in the elongation and desaturation of brain mitochondrial fatty acids. It is known that alterations in the ratio of saturated and unsaturated fatty acids affect membrane fluidity and by extension disrupt membrane integrity. If the integrity of the mitochondrial membrane is affected, then mitochondrial functions and brain bioenergetics could also be affected (Seyfried and Mukherjee 2005; Kiebish *et al.* 2008). Any changes in brain bioenergetics could affect neurodevelopment and brain function, and further suggest that there may be a link between abnormal mitochondrial fatty acid metabolism and the pathogenesis of ASD.

Possible role of alteration in brain lipids affecting gap junctional gating

One interesting pH dependent effect of PPA is its ability to rapidly and reversibly reduce intercellular coupling by the

closure of gap junctions (Rorig *et al.* 1996; Fujita *et al.* 1998). Gap junctions are composed of dodecamers, of a family of membrane proteins known as the connexins, whose assembly is profoundly affected by membrane fluidity. Gap junction coupling of the astrocyte network is involved in energy supply to neurons, buffering extracellular K^+ and glutamate and propagating calcium waves, all of which contribute to normal brain function and protect neurons from insults (Rouach *et al.* 2002). Pharmacological inhibitors of gap junctional gating have been shown to produce behavioral effects reminiscent of neuropsychiatric and mood disorders (Moore and Grace 2002; Juszczak and Swiergiel 2009). It has been shown that enrichment of astrocyte membranes with 22:6n3 increases gap junction coupling (Champeil-Potokar *et al.* 2006). In our model, a decline in 22:6n3 was generally associated with PPA infusion. In addition, the balance between saturates and unsaturates can influence the properties of proteins in the membrane and lipid signaling machinery, where the coupling capacity of the astrocytes is directly related to the proportion of saturated and unsaturated fatty acids present in their membranes. Changes in astrocyte membrane integrity may affect calcium homeostasis and protein phosphorylation on astrocyte membranes (Rouach *et al.* 2002; Champeil-Potokar *et al.* 2006). Our model shows that infusions of PPA alter the membrane composition of brain cells by altering the proportions of saturated and unsaturated fatty acids, including 22:6n3, present in the membrane. We speculate the alterations in brain membrane phospholipids may affect brain function by uncoupling gap junctions, and suggest that low gap junction coupling capacity may be associated with the pathophysiology of ASD.

The findings in this and previous studies from our laboratory offer further support of the validity of PPA infusion in rodents as a suitable model for ASD. Collectively, these studies offer further evidence that gut derived factors such as short chain fatty acids may be important as plausible environmental factors which can trigger or exacerbate ASD in sensitive subpopulations.

Conclusions

Abnormal lipid metabolism seems to be a common feature in ASD, considering several studies of the blood drawn from autistic patients indicate erythrocyte or plasma phospholipids and acylcarnitine profiles are altered in the disorder (Bell *et al.* 2000, 2004; Vancassel *et al.* 2001; Meguid *et al.* 2008; Wiest *et al.* 2009). Our rodent model indicates brain phospholipids and acylcarnitines are also altered following both BUT and PPA infusion which produced behavioral changes reminiscent of ASD. In most instances, the alterations in brain lipids were consistent with those observed in the blood from autistic patients.

Taken all together, infusions with enteric short chain fatty acids (PPA and BUT) induce ASD-like behavior in rodents and alter their brain lipid composition. The alterations in the brain lipid profile suggest possible disruption in membrane integrity affecting fluidity, signaling, uncoupling of gap junctions, consumption of plasmalogens possibly because of enhanced oxidation, and mitochondrial dysfunction affecting β -oxidation and bioenergetics. These seem to be plausible mechanisms that may be associated with the pathogenesis of autism and warrant further research.

Despite the promise of the PPA rodent model fulfilling some of the behavioral, electrographic, neuropathological and biochemical criteria for autism (Crawley 2004), caution needs to be exercised in suggesting PPA as a possible risk factor for the human condition. First, it should be noted that there are no studies directly measuring PPA in ASD patients. However, in addition to reported lipid profile findings, there is indirect evidence linking PPA to alterations in fatty acid metabolism (Wu *et al.* 1990), deficiencies in carnitine (Filipek *et al.* 2004), alterations in biotin (Zaffanello *et al.* 2003), and clostridial infections (Finegold *et al.* 2002a) in autism. There are similar metabolic trends noted following exposure to valproate, a teratogenic risk factor in autism (Schulpis *et al.* 2001). It is important to note that children suffering from propionic acidemia, a natural extension of the model presented, do not commonly present with symptoms of ASD. However, we would like to emphasize that our model is rather a study of brief, as opposed to continuous, brain infusions of enteric short chain fatty acids, including PPA, eliciting ASD like behavioral and brain changes in normal rodents.

We do not know if these PPA derived effects are causative or compensatory, or if there are regional differences in these profiles, similar to that found with our oxidative stress or neuropathological studies. In addition, it is not yet clear which of the diverse effects of short-chain fatty acids are responsible for the ASD-like behavioral or biochemical changes. Nevertheless, intraventricular administration of enteric short-chain fatty acids in rodents does show promise as an animal model to address the disparate behavioral and metabolic findings in ASD.

Acknowledgements

The authors would like to thank Francis Boon for his excellent technical assistance. The authors would also like to gratefully thank Dr Christopher Guglielmo, Department of Biology (UWO) for his contribution to data interpretation and the use of his laboratory space and equipment. This research was supported by contributions from Goodlife Children's Foundation and Autism Research Institute Foundation to Derrick MacFabe. We extend our heartfelt gratitude to David Patchell-Evans and Kilee Patchell-Evans. The authors declared no conflict of interest.

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