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Research report

Neurobiological effects of intraventricular propionic acid in rats: Possible role of short chain fatty acids on the pathogenesis and characteristics of autism spectrum disorders

Derrick F. MacFabe [a,*], Donald P. Cain [b], Karina Rodriguez-Capote [c], Andrew E. Franklin [d], Jennifer E. Hoffman [b], Francis Boon [d], A. Roy Taylor [d], Martin Kavaliers [b], Klaus-Peter Ossenkopp [b]

[a] *The Kilee Patchell-Evans Autism Research Group, Departments of Psychology and Psychiatry, Division of Developmental Disabilities, University of Western Ontario, Social Science Centre, Room 7252, London, Canada N6A 5C2*
[b] *The Kilee Patchell-Evans Autism Research Group, Department of Psychology and Graduate Program in Neuroscience, University of Western Ontario, London, Canada N6A 5C2*
[c] *The Kilee Patchell-Evans Autism Research Group, Departments of Psychology, Biochemistry and Obstetrics/Gynecology, University of Western Ontario, London Health Sciences Centre, London, Canada N6A 5C2*
[d] *The Kilee Patchell-Evans Autism Research Group, Department of Psychology, University of Western Ontario, London, Canada N6A 5C2*

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Abstract

Clinical observations suggest that certain gut and dietary factors may transiently worsen symptoms in autism spectrum disorders (ASD), epilepsy and some inheritable metabolic disorders. Propionic acid (PPA) is a short chain fatty acid and an important intermediate of cellular metabolism. PPA is also a by-product of a subpopulation of human gut enterobacteria and is a common food preservative. We examined the behavioural, electrophysiological, neuropathological, and biochemical effects of treatment with PPA and related compounds in adult rats. Intraventricular infusions of PPA produced reversible repetitive dystonic behaviours, hyperactivity, turning behaviour, retropulsion, caudate spiking, and the progressive development of limbic kindled seizures, suggesting that this compound has central effects. Biochemical analyses of brain homogenates from PPA treated rats showed an increase in oxidative stress markers (e.g., lipid peroxidation and protein carbonylation) and glutathione *S*-transferase activity coupled with a decrease in glutathione and glutathione peroxidase activity. Neurohistological examinations of hippocampus and adjacent white matter (external capsule) of PPA treated rats revealed increased reactive astrogliosis (GFAP immunoreactivity) and activated microglia (CD68 immunoreactivity) suggestive of a neuroinflammatory process. This was coupled with a lack of cytotoxicity (cell counts, cleaved caspase 3′ immunoreactivity), and an increase in phosphorylated CREB immunoreactivity. We propose that some types of autism may be partial forms of genetically inherited or acquired disorders involving altered PPA metabolism. Thus, intraventricular administration of PPA in rats may provide a means to model some aspects of human ASD in rats.

Keywords: Locomotor activity; Seizures; Dystonia; Kindling; Animal model; Oxidative stress; Neuroinflammation; Glutathione; Clostridia

1. Introduction

1.1. Autism spectrum disorders

Autism consists of a spectrum of traits which likely represent a group of neurodevelopmental brain disorders with a strong genetic basis. The abnormalities noted relate to development deficiencies of language and social interaction skills, appearance of repetitive and disordered movements [113], hyperactivity, sensory disturbances, restricted interests and sometimes self injury [3,190]. There is also an increased incidence of seizure disorder [15]. The prevalence of autism spectrum disorder (ASD) has increased over the last several decades, partly as a result of a broadening of diagnostic criteria and greater awareness among health professionals, but it is difficult to ascertain to what degree these factors account for the increase. Current

* Corresponding author. Tel.: +1 519 661 2111x84703; fax: +1 1519 661 3961.
E-mail address: dmacfabe@uwo.ca (D.F. MacFabe).

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estimates put ASD as high as four to six per thousand individuals [69]. As well, the prevalence is between four and eight times greater in males than in females [14]. The etiology of ASD is poorly understood but the disorder may be associated with a variety of other conditions, including fetal alcohol syndrome, prenatal exposure to thalidomide or valproate [112], fragile X syndrome [168], and tuberous sclerosis [175], amongst others [6,166].

Autism usually manifests in early infancy, characterized by an absence of age appropriate parental eye-contact or social development as well as abnormal movement patterns [164] based on faulty development of some reflexes. In about one-third of cases the onset of symptoms seems to be delayed until 2 years of age after apparent normal development [64]. Whether or not these later onset cases represent an acute regressive disorder, or the eventual manifestation of an ongoing disease process, has not yet been determined.

1.2. Genetic and environmental aspects of ASD

Clear evidence exists for a very strong genetic contribution to autism. A growing body of evidence from family and twin studies has shown a heritability estimate of 90% with a concordance rate of over 60% for monozygotic twins and close to 0% for dizygotic twins [53,65]. Linkage analyses have pointed to a complex multi-genetic cause of autism (e.g., [7,95,177,186]). However, it is also clear that environmental factors are likely of importance in the etiology of this disease spectrum as there is not complete concordance in monozygotic twins and there is evidence for epidemiological clustering [125,174].

There has been growing interest in the possible involvement of a variety of environmental agents, such as chemical toxins and infectious agents which could act during critical periods of pre and early postnatal development [91]. For example there is increased risk of ASD in children exposed prenatally to thalidomide, valproic acid, and ethanol [5]. These observations have led to the development of a number of animal models based on exposing rodents to thalidomide [112] or valproic acid [77] during the prenatal period. Other animal models have focussed on prenatal exposure to infectious agents such as Borna disease [84] and influenza viruses [59]. Anecdotal reports of autistic-like symptoms or enhanced symptoms, following acute gastrointestinal abnormalities [174] or upper respiratory infections, or vaccinations for viral infections [76] provide some additional evidence for putative environmental factors of interest. Clearly examination of the manner in which predisposing genetic factors might interact with environmental agents in the aetiology of ASD is a major challenge. Development of animal models for ASD allow for experimental examination of the effects of suspected environmental agents in a controlled dose-response manner.

1.3. Central nervous system and systemic involvement in ASD

Recent studies of human autopsy material have examined autism as a definable systemic disorder involving multiple pre- and post-natal factors that may affect brain development and function [5]. The brain tissue of autistics shows subtle developmental abnormalities, specifically in those areas concerned with language, movement, facial expression and social behaviour [44]. Individuals with autism may show enlarged brain size in the first few years of life, with altered migration of cortical, amygdalar and cranial nerve motor neurons, as well as cerebellar neurons [49]. Cell counts have shown that compared to controls, autistic brains contain smaller neurons with increased cell density in the amygdala, hippocampus, and granular cell layer of the cerebellar vermis [9]. Whether these abnormalities are due to altered neurogenesis [45], apoptosis [4], altered neural cytoarchitecture [125,138], or a combination of these factors, is not yet clear.

There are also some indications that autism may involve a disorder of glial cell function. Glial cells are of great importance in both the developing and mature nervous system with respect to cell–cell interactions during neural migration, and synaptic plasticity [16]. Glia form a functional syncytium necessary for the maintenance of a stable neural microenvironment especially during periods of increased metabolic stress [89]. Glial abnormalities may manifest as an overall increase in white matter thickness, a finding which has been observed in autistic patients [38]. Imaging studies have shown an increased thickness of the external capsule and increased water content in the white matter of autistics suggestive of an underlying glial disorder [73]. These observed abnormalities in brain morphology are accompanied by increased CNS immune activity. A recent study has found increases in reactive astrocytes and activated microglia in brains from autistics at autopsy, as well as the elevation of proinflammatory cytokines in the cerebral spinal fluid. These findings have been demonstrated both in young, as well as older patients suggesting that an inflammatory process may be present throughout the lifespan of autistic individuals [171]. Similar findings of heightened immune activity (i.e., increased Th2 cytokine levels) have also been demonstrated in peripheral blood monocytes from autistic patients [105].

Additional haematological studies have shown elevations in oxidative stress markers such as the inflammatory-related free radicals nitric oxide or hydrogen peroxide [155]. Platelet hyperserotonemia, and possible polymorphisms of the serotonin transport gene have been noted in 20–30% of patients [185]. Reductions in transferrin, ceruleoplasmin [40], or impaired glutathione metabolism [188] have also been observed, suggesting an overall impairment of a wide variety of metal or xenobiotic detoxification systems. However, it is unclear if these phenomena are causative, secondary, or even compensatory to some other inherited or acquired factor.

1.4. Gastrointestinal aspects of autism—anecdotal reports, enteric bacteria and diet-related phenomena

Gastrointestinal disturbances often co-exist with autism but have not been studied extensively. Some parents report an association between severe abdominal discomfort and the onset of autistic symptoms. Others report a worsening of their child's behaviour shortly after the ingestion of refined wheat and dairy

products, and a general improvement in behaviour following the elimination of these products from the diet [83]. In support of these reports, there is evidence in ASD of alterations of gut motility, increased permeability and intestinal lesions resembling, but not identical to, those observed in patients with gluten or casein allergy [178].

Lymphoid nodular hyperplasia with sporadic inflammation, particularly in the terminal ileum was noted in a group of 12 autistic patients with apparent regressive symptoms [174]. Reflux esophagitis, gastritis, duodenitis, reduced carbohydrate digestive enzymes, reduced duodenal paneth crypt cells, and hypersensitivity to secretin challenge [76] have also been noted. Blood mononuclear cells from autistic patients presenting with gastrointestinal inflammation show exaggerated cytokine production in response to gliadin and cow's milk protein [83]. These heightened immune reactions may indicate sensitivity to dietary proteins but it is unclear if these food components are causative or are the result of gastrointestinal inflammation.

These findings raise the possibility that some dietary or gut borne factor may influence brain function and symptomology in some patients. Moreover, a compromised gut–blood barrier may allow for systemic and CNS access to these and other compounds. However, no specific gut-derived factors have been identified to date which could provide a common explanation for both the behavioural and neuropathological changes observed in autism.

1.5. Propionic acid (PPA)—a possible environmental factor in autism?

Propionic acid (PPA) is an intermediary in cellular fatty acid metabolism found in high levels in the gut, along with a number of other short chain fatty acids, such as acetate and butyrate, each of which are a major metabolic end product of enteric bacteria [81]. In addition to its endogenous synthesis from amino and fatty acids [165], PPA is also present naturally in a variety of foodstuffs [187] and is commonly used as a food preservative that is added to refined wheat and dairy products [22].

PPA may play a role in the behavioural, neuropathological and biochemical abnormalities observed in autism. There are a series of inherited and acquired conditions which lead to elevations of PPA and other short chain fatty acids and these are related to developmental delay, seizure disorder and gastrointestinal symptoms, resembling some aspects of ASD [27,173]. Thus, PPA may be a putative link between dietary or enterobacterially derived metabolites along with genetic predisposition, and subsequent features of ASD.

Being a weak organic acid, PPA exists in ionized and non-ionized forms at physiological pH allowing it to readily cross lipid membranes, including the gut–blood and blood–brain barriers [86]. In addition, PPA and related short chain fatty acids are taken up by monocarboxylate receptors in the gut lumen [163] and cerebrovascular endothelium [13], as well as neurons and glia [101,127] where they are thought to comprise a major energy source in brain metabolism, particularly during early brain development [136].

PPA has a number of direct effects on gastrointestinal physiology. Along with acetate and buyrate, PPA is known to reduce gastric motility and increase the frequency of contractions, presumably via a reflex that involves direct contact of these short chain fatty acids with the terminal ileum [51]. In addition, PPA increases contraction of colonic smooth muscle [103], dilates colonic arteries [108], activates mast cells [85] and increases the release of serotonin from gut enterochromaffin cells [104]. Thus PPA is in a position to interfere with normal gastrointestinal peristaltic activity and cause inflammation that is to some degree reminiscent of the gastrointestinal dysfunction observed in some patients with ASD [76].

The manner in which increased systemic PPA levels may influence the central nervous system are unknown. *In vitro* and animal studies suggest that increased levels of PPA affect diverse processes, including Na+, K+-ATPase activity [183], NMDA receptor activity [57], cytoskeletal phosphorylation [55], intracellular calcium levels [110], scavenging of reactive oxygen and nitrogen species [10,81], and modulation of gap junctions [143].

Increased PPA levels may interfere with overall cellular metabolism. One of the mechanisms by which this might occur is via the uncoupling of mitochondrial function, via direct inhibition of oxidative phosphorylation [20]. Other effects could include sequestration of carnitine [19] and increasing the levels of propionyl coenzyme A levels which could result in an inhibition of short chain fatty acid oxidation. Elevated PPA could also produce sensitivity to oxidative stress which could result in an increase in damage caused by other environmental toxic factors (e.g., hydrocarbons, metals) or infectious agents [173].

Elevated PPA may also modulate immune function by stimulating the release of proinflammatory cytokines such as interferon (IFN)-gamma [39]. This immune system modulation may also occur via the direct activation of G-protein coupled receptors specific to short-chain fatty acids on polymorphonuclear leukocytes and neutrophils [24,92]. Activation of these receptors leads to alterations in intracellular calcium levels and cellular motility [24] which may promote the migration of immune cells to areas, such as the digestive tract, where PPA levels are high. However, it is unknown whether a similar immune system activation by PPA occurs in the CNS. Evidence from *in vitro* studies suggest that a variety of cells in the gut [58], the immune system [24], and the CNS [143] can concentrate PPA and other weak organic acids leading to intracellular acidification, a phenomena which can be exacerbated with additional minor reductions of pH in the extracellular environment [86]. This may raise the possibility that in clinical conditions of elevated PPA, serum levels might not be reflective of intracellular PPA levels.

One interesting effect of PPA is its ability to reversibly reduce intercellular electrotonic coupling via the closure of gap junctions, presumably by inducing intracellular acidification [67,143]. Gap junctions are intercellular channels composed of connexin proteins which are gated by a number of factors that are elevated by PPA including dopamine [142,146,172], calcium [130], nitric oxide [114] and cytokines [87]. Gap junctional coupling is integral for the synchronization of neural electrical activity within discrete functional groups [93], and this

coupling is more extensive during early brain circuit formation and development [127]. In addition, gap junctions are extensive between glial cells forming a functional neuroprotective syncytium [2,129,140] which protects neurons during periods of increased metabolic stress by the uptake and intercellular diffusion of glutamate and potassium.

Gap junctional communication is involved in neurotransmission in those areas implicated in seizure development and movement disorder, including the basal ganglia [119], prefrontal cortex [142], nucleus accumbens [120] and the hippocampus [107,142]. Intrastriatal injections of known gap junction blocking agents, such as carbenoxelone and anandamide, produce stereotypical movements, increase locomotion and disruption of motor sequencing in rodents [107]. Additional studies of gap junction knockout mice show abnormal brain development [68], exaggerated responses to neurotoxic insults [111], and abnormal behaviour [66].

1.6. PPA and enteric bacteria

The human digestive tract is host to a wide variety of intestinal bacterial florae, both harmful and protective, that produce a number of metabolic products capable of entering the systemic circulation in both normal and pathological conditions [184]. Many of these bacteria produce a number of short chain fatty acids, such as acetate, butyrate, and PPA, via the break down of carbohydrates, and amino acids [52].

Of particular interest are the Clostridia, a family of heterogeneous anaerobic, spore forming Gram-positive rods. Clostridia are major gut colonizers in early life and many of which are producers of PPA and other short chain fatty acids [159]. *Clostridium difficile* is known to be a major cause of severe gastroenterological diseases such as pseudomembranous colitis, but may also be a major cause of antibiotic associated diarrhoea, both pre- and post-natally [61]. This pathogen is known to produce an enterotoxin A, primarily responsible for gastrointestinal symptoms through mucosal damage and lymphocyte infiltration, and cytotoxin B. However, the exact mechanism by which this gut pathology is produced is unknown [61]. Antibiotic resistant clostridial strains play a role in a wide variety of hospital and community acquired infections [102] in adult patients, but their role in paediatric diarrhoea related to antibiotic treatment has not been extensively studied [61]. Spore forming anaerobes and microerophilic bacteria, particularly from clostridial species, have been shown to be elevated in late-onset autistic children but absent in controls [64]. As well, PCR analyses of stool samples from patients with regressive autism have revealed increases in clostridial species including *C. difficile* [157]. The eradication of the pathogen with oral vancomycin treatment has improved symptoms in some patients [157].

PPA is also produced by propionibacteria of the intestinal tract, largely from bovine sources [81], as well from endogenous bacteria in skin [189] and oral mucosa [18]. It is intriguing to speculate about the possible interaction of both environmental and genetic factors which could increase PPA over the developmental time span and play a potential role in the development or exacerbation of autism or other neurodevelopmental disorders.

1.7. Genetic and acquired disorders involving PPA

There are a number of genetic and acquired conditions with CNS involvement where elevated levels of PPA are known to exist. Most notable is propionic acidemia, a genetic neurodevelopmental disorder of amino and fatty acid metabolism resulting in developmental delay, seizure development, movement disorder and gastrointestinal symptoms [60]. Patients often present with life threatening illness during the neonatal period characterized by vomiting, severe metabolic acidosis and hyperammonemia. Neurological symptoms include developmental delay, seizure, choreoathetoid movements and dystonia [56].

Other patients may present later in life with varying severities of the disorder, often without observable systemic metabolic complications [43,117]. Results of neuroimaging with magnetic resonance spectroscopy or positron emission tomography [1,41] and neuropathological [72] analyses of the disorder, are also variable. Lesions, including astrogliosis, Alzheimer type II astrocytes, mild cell losses in the caudate, putamen, globus pallidus and cortical structures and associated endothelial damage and diffuse white matter changes have been observed in these patients. To our knowledge, there are no studies which have examined neuropathological changes in the brains of patients with propionic acidemia using modern immunohistochemical techniques. The mechanisms responsible for the variable clinical presentation of cognitive impairment, intermittent seizure and movement disorder, often with no correlation to systemic illness [117], are unknown.

Propionic acidemia is a potentially treatable condition. Primary means of treatment include reduction of PPA and amino acid intake in the diet and intermittent eradication of PPA producing bacteria via metronidazole and gut motility agents [134]. Supplementation with carnitine to promote PPA and other fatty acid metabolism [97] offers an additional treatment option for this condition. Propionic acidemia is caused by deficient activity in either one of two non-identical subunits of the biotin-dependent enzyme propionyl CoA carboxylase (PCC) [128]. This mitochondrial enzyme is responsible for the breakdown of PPA and other short chain fatty acids, as well as a number of amino acids.

PPA and other short-chain fatty acids are elevated in the related metabolic disorder of methylmalonic acidemia [98], as well as in disorders of biotin metabolism [123], B12 deficiency [21], during valproate therapy [148], and following ethanol consumption [92]. In each of these conditions, PPA and other fatty acid levels may potentially rise to millimolar levels in the serum, but exact CNS levels are unknown [143].

Given the diverse biological properties of PPA, it was suggested that the developing nervous system, either pre- or post-natally, might be sensitive to elevated levels of this short chain fatty acid. Intraperitoneal injections of PPA between post-natal days 16 and 28 of rats has been used as a possible model for human propionic acidemia, and has been shown to produce a number of biochemical [26] and behavioural [25] effects resembling this disorder. The behavioural effects include delayed eye opening, impaired free fall righting, lack of habituation to an open field, and a lack of retention to a shuttle avoidance task [25]

and impaired spatial performance in a water maze [131]. There also are alterations in neural cytoskeletal [161], mitochondrial [99] and lipid profiles [167].

To date, there have been no detailed studies of the behavioural, electrophysiologic, biochemical and neuropathological effects of brief or chronic microinfusions of PPA directly into the central nervous system. We hypothesized that increased exposure to elevated intraventricular levels of PPA may result in hyperactivity, repetitive movements, and development of seizures often seen in ASD patients [169,178]. Here we report preliminary findings from studies which have examined the effects of acute or chronic CNS administration of PPA to adult rats.

2. General materials and methods

2.1. Animals and housing facilities

A total of 74 adult male Long–Evans were used (Charles River Laboratories, Quebec, Canada). In each of the studies, rats weighed approximately 300 to 350 g (approximately 75 days old) at the time of surgery. Animals were individually housed at a controlled temperature (21 ± 1 °C) with *ad libitum* access to food (Prolab rat chow) and water. All behavioural testing occurred during the light phase of a 12:12 h light:dark cycle (lights on 07:00 to 19:00 h) under normal lighting conditions. The rats were naive to all experimental procedures prior to surgery. Procedures were completed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and approved by the University of Western Ontario Animal Use Committee.

2.2. General surgical procedures for cannula implantation

Animals in each experiment were implanted with a 23 gauge guide cannula using standard stereotaxic techniques. Rats first received atropine pretreatment prior to all surgical procedures. In experiment 1, sodium pentobarbital anesthesia (60 mg/kg i.p.) was used whereas animals in experiments 2 and 3 were anaesthetized using inhaled isoflurane and oxygen. Animals were placed in a stereotaxic apparatus and body temperature was maintained at normothermia using heating pad. Surgical procedures and introduction of the guide cannula and electrodes were carried out under aseptic conditions. The tip of the guide cannula was placed immediately below the border of the corpus callosum into the lateral ventricle (AP 1.3 mm, ML 1.8 mm; [126]). The tip of the 30 gauge injection cannula protruded .5 mm beyond the tip of the guide cannula to allow compound infusion into the lateral ventricle. The cannula was sealed with an obtruator, which was removed just before each injection. Small screws were placed in the top of the skull and the screws and cannula were affixed to the skull with dental acrylic. Testing procedures in each experiment began approximately 14 days after surgery.

2.3. General procedures for intracerebroventricular (ICV) infusions

In all studies, compounds were infused using a 30 gauge injection cannula attached to a Sage syringe pump with sterile PE10 tubing. Infusions took place over a 60 s period. The infusion cannula was allowed to remain in place for an additional 60 s before being removed.

3. Experiment 1—Effects of PPA on electrographic activity and behaviour

In this study we examined the electrographic and behavioural effects of repeated, spaced intraventricular infusions of PPA and structurally similar compounds in freely moving rats. Electrographic responses were recorded from chronically implanted electrodes in neocortex, hippocampus and striatum, to determine the possible relation of behaviour to electrographic activity in these same rats. The prime goal was to evaluate and quantify the behavioural effects, including convulsive behaviour, in relation to electrographic activity. A second goal was to examine the permanence of any behavioural and/or electrographic responses to PPA.

3.1. Surgery—electrode implantation

In addition to the guide cannula, animals were stereotaxically implanted with three monopolar recording electrodes (127 μ Nichrome wire insulated with Teflon except at the cut tips) placed into frontal cortex (AP +3.2, ML 3.0, 2.0 mm below skull surface), dorsal hippocampus (AP 4.0 mm, ML 2.5, 3.0 mm below skull surface), and caudate nucleus (AP +0.7 mm, ML 2.2, 5.0 mm below skull surface) in the hemisphere ipsilateral to the guide cannula. The reference for recording consisted of a length of Nichrome wire soldered to a skull screw, which was placed in the nasal sinus. A similar wire attached to a screw placed over posterior cortex served as a ground that was attached to the polygraph chassis. Miniature connectors allowed the rat to be connected to the inputs of a Grass polygraph using individually shielded flexible wire leads, with the shielding connected to ground. Small screws were placed in the top of the skull and the cannula, recording electrodes, and connectors were affixed to the skull with dental acrylic.

3.2. Treatment groups

Rats were randomly allocated to the following groups: low PPA (4.0 μl of a 0.052 M solution, $n = 8$); high PPA (4.0 μl of a 0.26 M solution, $n = 8$); propanol control (4.0 μl of a 0.26 M solution, $n = 7$); sodium acetate control (4.0 μl of a 0.26 M solution, $n = 6$) and PBS control (4.0 μl PBS, $n = 9$). Effective doses of PPA were selected on the basis of unpublished pilot work involving repeated, spaced infusions with behavioural monitoring. Compounds were dissolved in PBS vehicle and buffered to pH 7.5 using HCl or NaOH.

3.3. Experimental procedures

Rats were first habituated to the testing box (clear Plexiglas, 40 cm × 40 cm × 30 cm) for three 15 min sessions on different days without infusion or recording. The next two sessions served as baseline recording sessions lasting 30 min each with leads connected for behavioural monitoring and electroencephalographic (EEG) recording in the absence of any infusions. Pilot work with repeated daily infusions of PPA indicated that the major changes in responsivity took place during the first five infusions, and the majority of behavioural effects occurred within 30 min of infusion. Therefore, rats received a series of five test sessions during which compounds were injected. The first 10 min of each of these sessions, prior to infusion of compounds, served as a habituation period with the cannula and leads connected, and with behavioural monitoring and EEG recording. The intraventricular infusion was then made, followed by behavioural monitoring and EEG recording for a further 30 min. Convulsive behaviour was scored using the Racine five-point scale [135]. A dual camera arrangement allowed for simultaneous videotaping of behaviour and EEG tracings using a split-screen technique. The videotapes were scored offline by an observer blind to group membership for abnormal behaviours which included retropulsion, hyperextension, turning, and dystonia (see Section 3.7). Based on pilot work and previous kindling research [29] all sessions were spaced at 48–72 h intervals to allow recovery from any epileptic events and clearance of the injected compounds.

3.4. Permanence and crossover testing

After completion of initial testing rats were allowed a 3 week period with no testing followed by further testing with either the same compound used during the initial testing phase, to determine the permanence of any potentiated response, or a crossover test with pentylenetetrazol (PTZ, 1.0 mg in 4.0 μl sterile saline, i.c.v.), a standard chemical convulsant treatment that kindles seizures when infused in a spaced, repeated manner [29]. Testing was continued as described previously until either a stage 5 convulsion occurred or five additional infusions had been given.

3.5. Tissue preparation and histology

After the last test session rats were deeply anesthetized with sodium pentobarbital and perfused transcardially with ice cold 0.1 M phosphate buffered saline (pH 7.5, PBS) followed by 4% paraformaldehyde in PBS. The brain was removed from the skull and cryoprotected in 18% sucrose in PBS. Serial coronal 40 μm thick brain sections were cut with a cryostat along the cannula and electrode tracks, then mounted on glass slides, dehydrated with increasing concentrations of ethanol and xylenes using standard histological procedures, and stained with cresyl violet for Nissl substance to allow confirmation of cannula and electrode placements.

3.6. Statistical analyses

Data were analyzed using one-way and mixed design analysis of variance (ANOVA) procedures with Newman–Keuls post hoc pair-wise tests for group differences where appropriate.

3.7. Results and discussion

3.7.1. Histology

Four rats had misplaced guide cannulae and were eliminated from further analyses; one rat from the low PPA group (leaving $n = 7$), one from the sodium acetate control group ($n = 5$), and two from the PBS Control group ($n = 8$). All other rats had cannulae and electrodes accurately placed in the intended structures. Gross histological structures were unremarkable in all groups and showed no evidence of diffuse neuronal loss in cortex, hippocampus and basal ganglia (see Fig. 1).

3.7.2. Epileptic and convulsive responses

All groups, including controls, displayed some degree of epileptic responsivity but differed widely in the nature and degree of response. Epileptiform spiking was evident in all rats in both the low and high PPA groups, with clear evidence of progression in both electrographic (EEG) spiking and accompanying convulsive activity. As shown in the EEG records in Fig. 2A, epileptiform activity typically appeared first in the hippocampal trace, followed some seconds later by epileptiform activity in the neocortical and/or caudate traces. During subsequent sessions epileptiform activity strengthened in all three sites (see Fig. 2B and C). Epileptiform activity during initial sessions was usually accompanied by either no convulsive behaviour or Stage 1 convulsions, with progressively stronger convulsions in subsequent sessions, culminating in Stage 4 or 5 convulsions in most rats in the high PPA group (see Fig. 2). Spike frequency increased across sessions, as exemplified in the records shown in Fig. 2 in which maximal spike frequency in hippocampus increased from approximately 3.8 Hz during session 1 (Fig. 2A) to approximately 6.1 Hz during session 4 (Fig. 2C). Epileptiform responses were seen in all rats injected with PPA, but responses were stronger and of longer duration in the high PPA group compared to the low PPA group (see Fig. 3B and C).

The epileptiform spiking seen in most control rats was typically weak, sporadic, and of short duration (see Fig. 3). Brief epileptiform spiking occurred in 3 of the 15 rats in the propanol and PBS control groups, at long latency and on a total of 6 of the 75 initial test sessions given to these rats. There was no epileptiform spiking or convulsive behaviour during the other 69 initial test sessions in these rats. Epileptiform spiking occurred in all five rats in the sodium acetate control group, with stages 0 or 1 convulsion scores in four rats and a brief stage 4 convulsion in the 5th rat. The short latency to the onset of spiking, which began before the completion of the injection in 56% of the cases, and the sporadic nature and short duration of the spiking suggests that these responses may have been pressure induced, as has been observed previously [46]. There was no evidence of progressive strengthening of electrographic seizures or convulsions in any control rat.

Statistical analysis confirmed the impressions described above. ANOVA indicated significant group differences in the three measures reported in Fig. 3 (latency: $F(4,29) = 15.54$, $p < .0001$; maximum convulsion: $F(4,29) = 6.10$, $p < .001$; duration: $F(4,29) = 5.51$, $p < .002$). Post hoc pair-wise comparisons (Newman–Keuls) indicated a dose-response effect of PPA in two of these measures (maximum convulsion: high PPA versus low PPA, propanol control, and PBS control, $p < .05$; sodium acetate control versus propanol control, $p < .05$, Fig. 3B; duration: high PPA versus all other groups, $p < .05$), Fig. 3C. The low and high PPA and sodium acetate control groups had shorter latencies to onset of epileptiform spiking than the propanol and PBS control groups ($p < .05$, see Fig. 3A).

To further evaluate the progression of responses during initial kindling sessions 1–5, maximal convulsion stage reached during each session was plotted as a function of session number (see Fig. 4). ANOVA indicated a dose-response effect of PPA and a progressive increase in convulsion stage across test sessions (group: $F(1,13) = 11.4$, $p < .005$; trials: $F(4,52) = 3.21$, $p < .02$). These results are consistent with a kindling effect due to the repeated, spaced infusion of PPA.

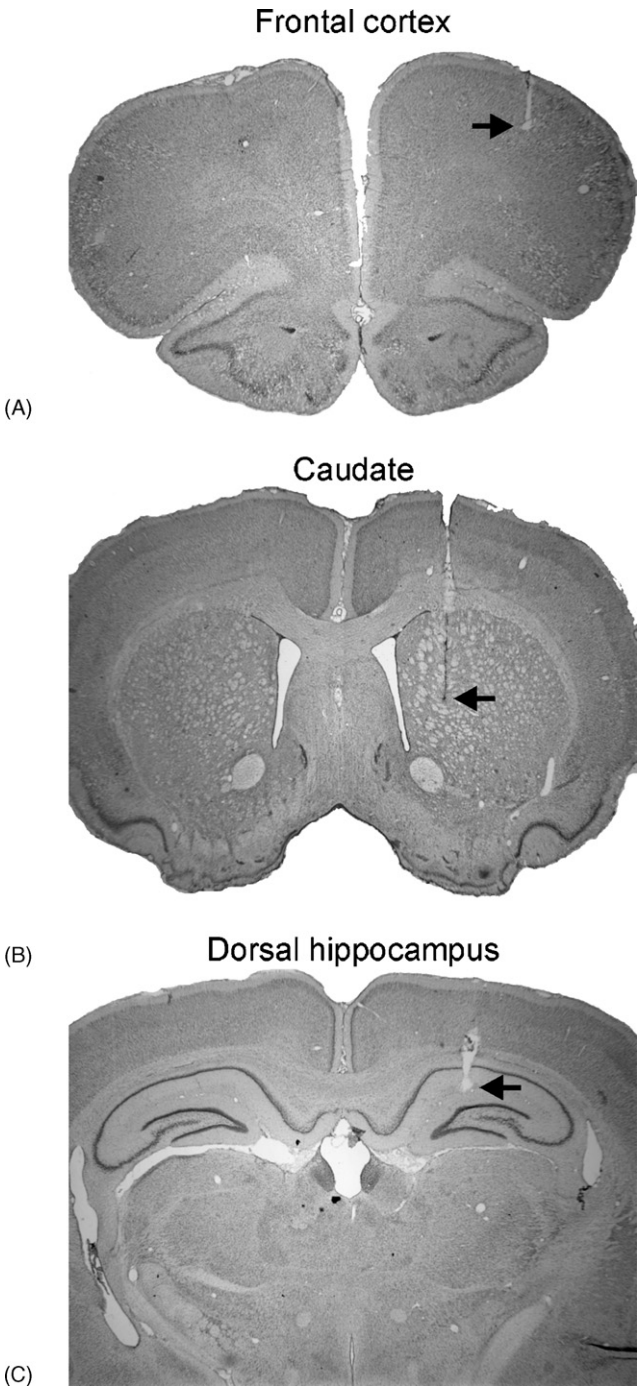

Fig. 1. Coronal sections of Nissl stained brains showing typical locations of recording electrode tips in frontal cortex (A), caudate nucleus (B), and dorsal hippocampus (C). Arrows indicate the tip of each recording electrode.

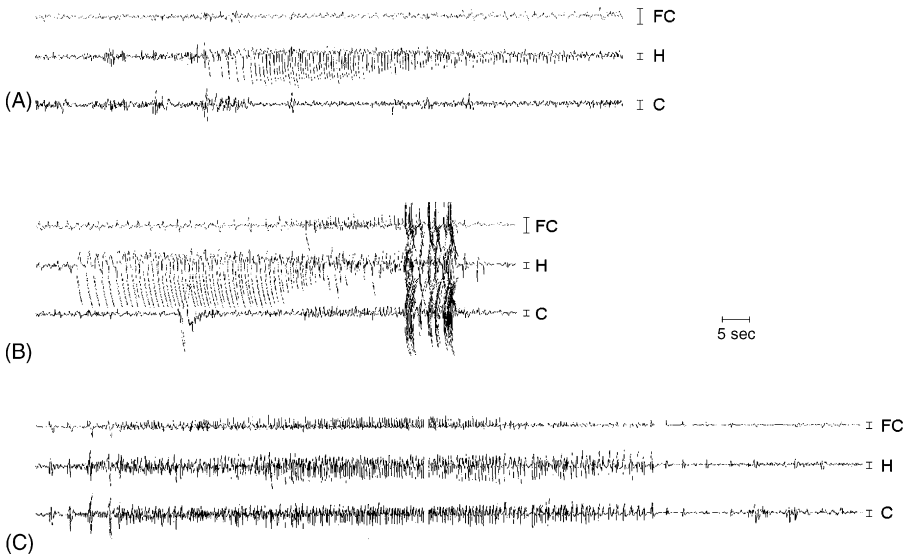

Fig. 2. Kindled limbic seizures in response to repeated infusions of PPA: representative electrographic seizure records from Rat AUS25 in the high PPA group. (A) Session 1, seizure activity in dorsal hippocampus, convulsion stage 0. (B) Session 2, seizure activity in frontal cortex and dorsal hippocampus, convulsion stage 2. (C) Session 4, seizure activity in frontal cortex, dorsal hippocampus, and caudate, convulsion stage 4. Spike frequency and seizure duration increased from A to C. C: caudate; FC: frontal cortex; H: dorsal hippocampus. Amplitude calibration: 50 μv; time marker: 5 s.

3.7.3. Abnormal behavioural responses

In addition to the results described above, 14 of the 15 rats given PPA injections displayed a variety of abnormal behavioural responses, in some cases associated with single epileptiform spikes or short, distinct bouts of epileptiform spiking that were not accompanied by limbic convulsion behaviours. Four prominent abnormal behaviours occurred: retropulsion (dorsiflexed spine with repeated forelimb extension, pushing the body backward), snake like posture (hyperextension of body parallel to the floor, usually with paddling motions of the limbs), turning (full body turning in a continuous rotating motion in place or in a limited area of the test box; 1 turn = 360° of body rotation), and limb dystonia (dystonic movement of forelimb or hindlimb contralateral to the injection, usually with repeated adduction and extension).

Frequencies of the abnormal behaviours were scored during the two 30 min baseline sessions and during the five initial test sessions for 30 min beginning at the completion of the intraventricular infusion and are shown in Figs. 5 and 6. One or both doses of PPA increased the frequency of all behaviours whereas, with one exception, none of the control treatments altered the frequency of the behaviours. The exception was treatment with sodium acetate, which increased the frequency of turning (see Fig. 5). These impressions were confirmed by analyses indicating increased retropulsion by the low PPA group ($F(4,34) = 7.67$, $p < .0001$; low PPA versus all control groups, $p < .05$), increased snake posture by the low and high PPA groups ($F(4,34) = 7.05$, $p < .0001$; low and high PPA versus all control groups, $p < .05$), and increased turning by the high PPA and sodium acetate groups ($F(4,34) = 14.57$, $p < .0001$; high PPA and sodium acetate groups versus all other groups, $p < .05$.

Detailed analysis of group means relating to the short bouts of epileptiform spiking accompanied by limb dystonia indicated that these occurred with equal frequency in response to infusion of PPA at either low or high doses, but did not occur in response to infusion of any of the control substances ($F(4,31) = 7.4$, $p < .0001$; low PPA and high PPA versus all other groups, $p < .05$; see Fig. 6A). Fig. 6B shows plots of the frequency of the short bouts of epileptiform spiking with dystonia as a function of test session. Analysis of the frequency of bouts failed to yield group or trial effects ($p > .05$ for both) suggesting that the occurrence of the bouts of epileptiform spiking with dystonia did not vary with dose of PPA and did not systematically increase with repetition of the PPA infusions. This outcome was consistent with the fact that seven of seven low PPA rats displayed bouts of epileptiform activity with dystonia during test sessions prior to the test session during which the first kindled convulsion of stage 1 or above occurred (compare Figs. 4 and 6; see Fig. 7A, D and F). Most rats in the high PPA group displayed their first bouts of epileptiform activity with dystonia and their first kindled convulsion during the same test session, but in no case did these events overlap in time (see Fig. 7F).

Limb dystonia and retropulsion occurred coincident with epileptiform activity, examples of which appear in Fig. 7. Brief forelimb or hindlimb dystonia occurred coincident with single epileptiform spikes in the caudate EEG trace (Fig. 7B and E). Limb dystonia, at times alternating with retropulsion, also occurred coincident with short bouts of epileptiform spiking (Fig. 7A, D and F). Several observations suggested that single spikes or short bouts of epileptiform spiking in the caudate nucleus, but not in frontal cortex or dorsal hippocampus, were important for the behaviours. First, limb dystonia or retropulsion always occurred coincident with single epileptiform spikes or short bouts of epileptiform spiking in the caudate (see Fig. 7A, B, and D–F). Second, frontal cortex or dorsal hippocampal spiking was neither necessary nor sufficient for the behaviours to occur (see Fig. 7A–D). Third, single spikes in the caudate record that were accompanied by coincident brief limb dystonia invariably led spikes in frontal cortex by approximately 500 ms and led spikes in hippocampus by approximately 75 ms (see Fig. 7A, B, D, and E).

3.7.4. Permanence and crossover testing

After the completion of initial testing and the 3 weeks rest period rats received additional infusions of the same compound at the same dose used during initial testing, or were crossed over to PTZ. Results from the rats in the low or high PPA groups tested for the permanence of the response to PPA ($n = 4$ and 4, respectively) are presented in Fig. 4, which indicates no decrease in the response to PPA after the 3 weeks rest period. Propanol control rats retested with propanol ($n = 4$) displayed no spiking or convulsive behaviour, and PBS control rats retested with PBS ($n = 2$) displayed only sporadic and brief spiking comparable to that seen during initial testing (data not shown). Sodium acetate control rats retested with sodium acetate ($n = 3$) all displayed epileptiform spiking and behavioural convulsions that were comparable to or stronger than the responses that occurred during initial testing (data not shown).

Due to the limited number of rats crossed over to PTZ, rats in the low and high PPA groups that were crossed over to PTZ were combined ($n = 6$) and compared to rats in the propanol and PBS control groups that were crossed over to PTZ ($n = 8$).

The maximal convulsion stage reached by the low and high PPA rats given PTZ (stage 3.2 ± 0.73, mean ± S.E.M.) was greater than the maximal convulsion stage reached by the propanol and PBS control rats given PTZ (stage 0.37 ± 0.18; $F(1,11) = 24.56$, $p < .001$). This suggested that initial kindling with PPA led to a facilitation, or transfer [31], of the kindled response to PTZ. Only one sodium

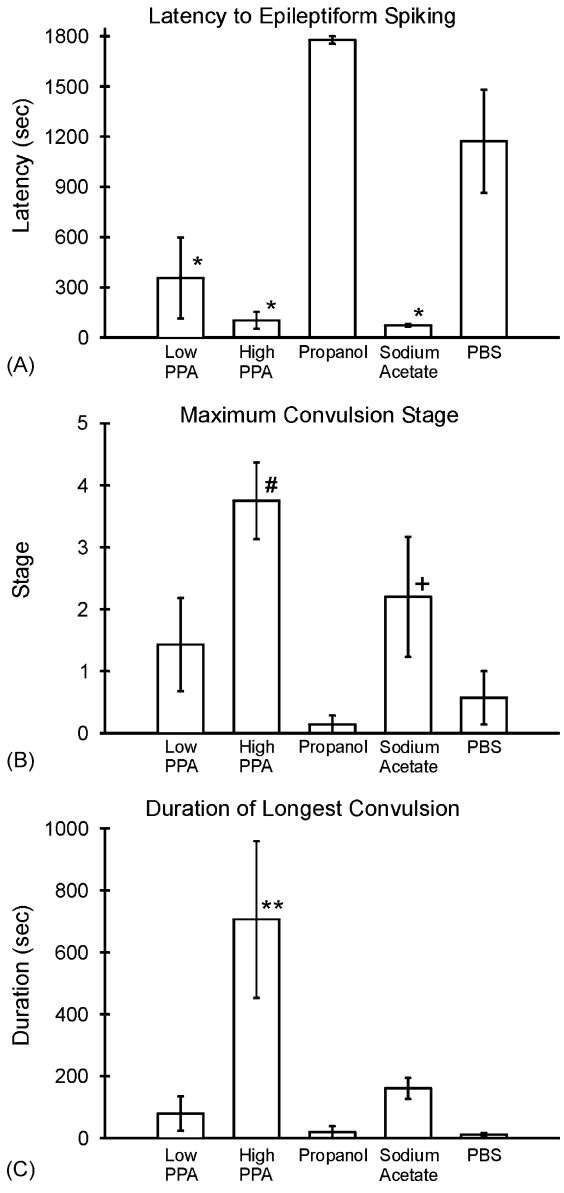

Fig. 3. Kindled seizure manifestations in response to repeated infusions of propionate: group mean (±S.E.M.) data summed across the five initial testing sessions. Latency to epileptiform spiking was measured from the end of the intraventricular infusion to the start of a seizure. Maximum convulsion stage was rated using the Racine [135] kindling scale. Duration of the longest convulsion was of the longest continuous convulsion during a session. *Different from propanol and PBS controls; #Different from low PPA, propanol control, and PBS control; +different from propanol control; **Different from all other groups.

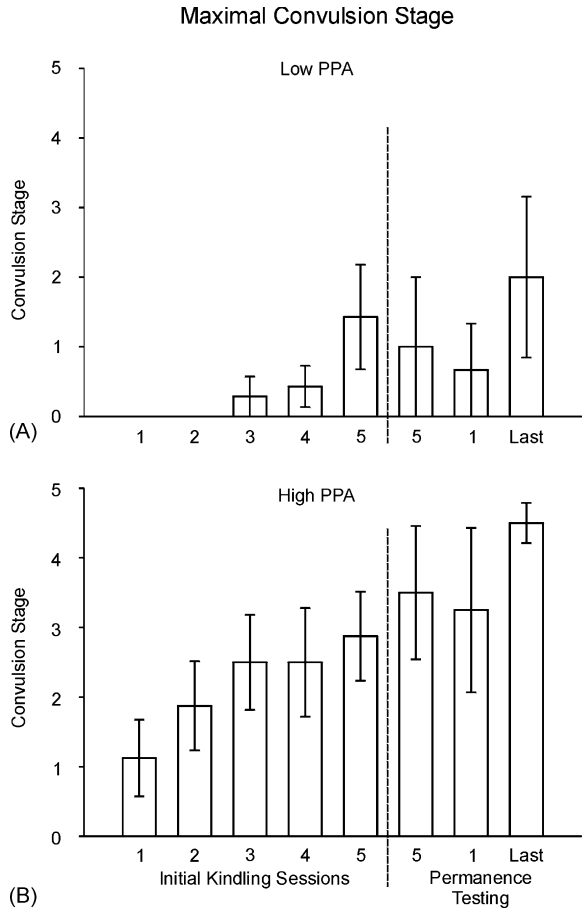

Fig. 4. Kindled convulsion stages during initial kindling and permanence testing with PPA: group mean (±S.E.M.) data for low and high PPA groups. Maximal convulsion stage was rated using the Racine [135] kindling scale. Both PPA groups exhibited increases in convulsion strength during initial testing, but the high PPA had more severe convulsions than the low PPA group. A 3-week rest interval is represented by the vertical dashed lines. Because only a subset of rats in each group was tested for permanence of kindled convulsions after the rest interval, the convulsion on the 5th session of initial testing for each subset is shown for comparison purposes (bars labeled '5' during permanence testing). The convulsions during permanence testing remained as strong as those at the completion of initial testing.

acetate control rat completed crossover testing to PTZ. In this rat the responses to both sodium acetate and PTZ were comparable (maximum convulsion stage displayed to each compound: stage 1; maximum seizure duration: sodium acetate, 160 s; PTZ, 130 s).

Crossover testing to PTZ provided an opportunity to compare the incidence of caudate epileptiform bouts with dystonia in response to PPA or PTZ in the same rats ($n = 6$). The mean incidence of epileptiform bouts with dystonia per session in response to PPA prior to crossover was 2.8 ± 1.2. Only three of six PPA rats crossed over to PTZ displayed a bout of spiking with dystonia in response to PTZ, and the overall mean incidence of bouts per session in response to PTZ was 0.3 ± 0.2 (paired t-test, PPA versus PTZ bouts, $t(1,11) = 2.3$, $p < .04$). This suggests that the PPA response of caudate epileptiform bouts with dystonia crossed over to PTZ in a weak and limited manner only.

A main finding of this study was that repeated, spaced administration of PPA, but not the control compounds 1-propanol or PBS vehicle, resulted in the progressive development of limbic-type kindled seizures. A similar, rapidly induced but shorter acting electrophysiological and behavioural response occurred with isomolar administration of sodium acetate. A second finding was that administration of PPA, but not control compounds, led to abnormal behaviours including turning, snake-like posture, retropulsion, and limb dystonia, the latter two behaviours always occurring coincident with sharp wave electrical activity in the caudate nucleus.

Several of these findings are consistent with results of previous kindling research using chemical kindling treatments [29,30,32,132]. These include: (1) the speed of development and ultimate kindled convulsion stage that was reached were related to the dose of PPA that was administered; (2) the kindled seizures were persistent, as evaluated after a 3-week rest period followed by further injections of PPA; (3) kindled seizure susceptibility transferred to the convulsant PTZ infused after a 3-week rest period. The gradual development of epileptiform spiking in the hippocampus together with gradual progression through Racine's limbic convulsion stages across sessions is consistent with a limbic origin for the kindled seizures [28]. The close proximity of the dorsal hippocampus to

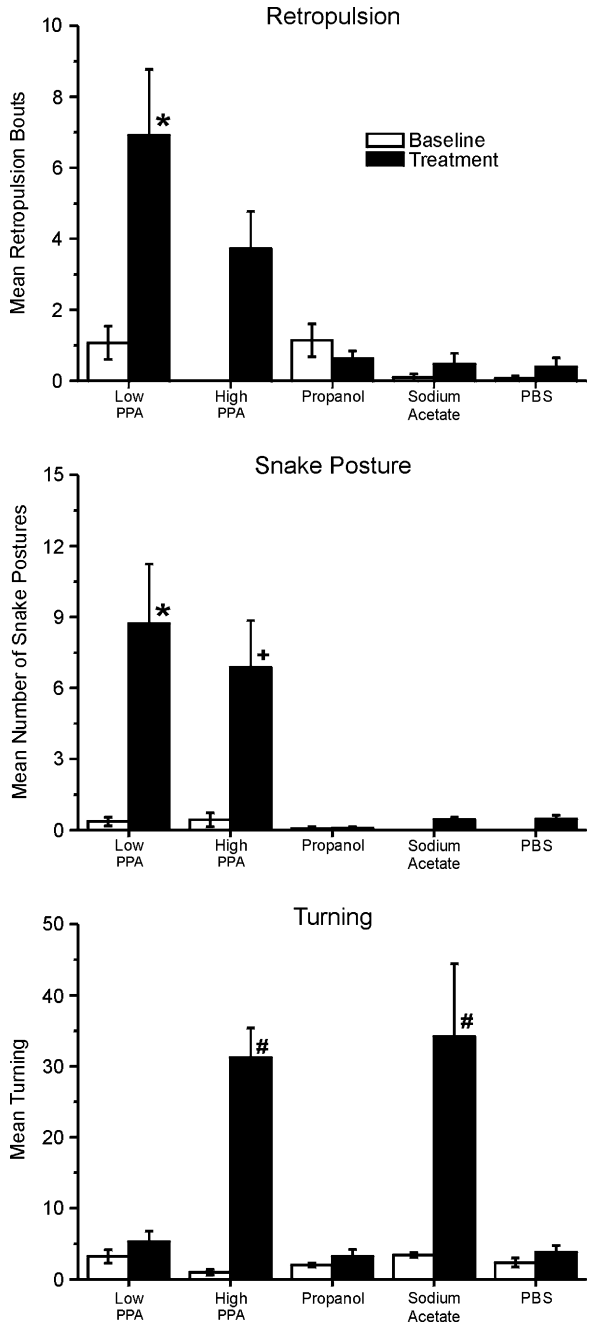

Fig. 5. Abnormal behaviours in response to ICV infusions: group mean frequency (±S.E.M.) of behaviours per baseline or initial test session. See text for definitions of the behaviours. Either one or both doses of PPA increased the abnormal behaviours relative to control treatments. With the exception of sodium acetate, which increased turning, no control treatment increased abnormal behaviour. $^{*}p < .05$ or better vs. all control groups; $^{\#}p < .05$ or better vs. low PPA, propanol control and PBS control.

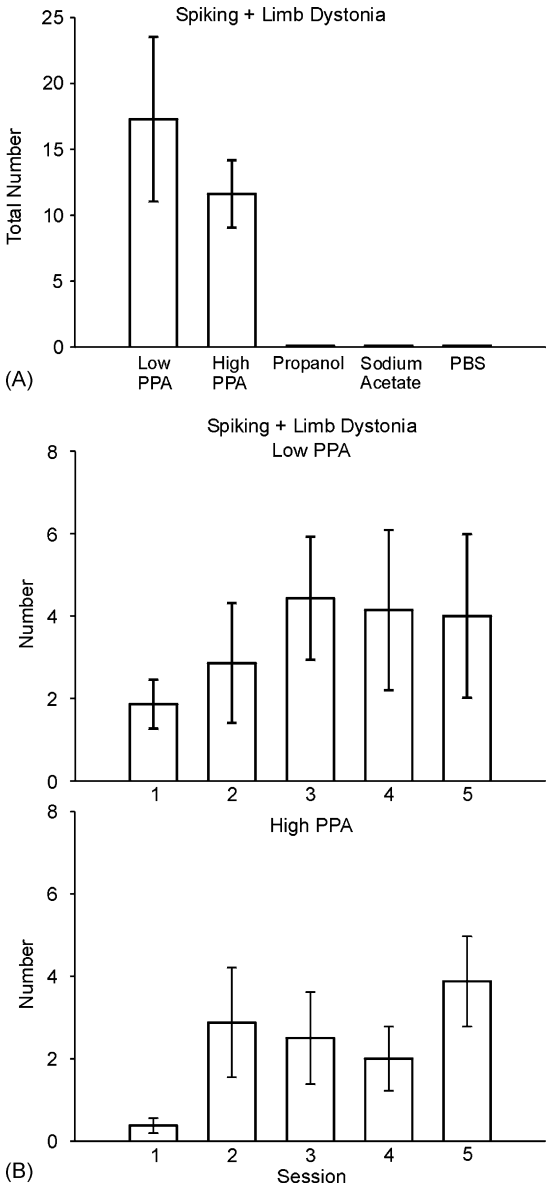

Fig. 6. Group mean (±S.E.M.) incidence of short bouts of epileptiform activity and limb dystonia. (A) Number of bouts of epileptiform activity and accompanying abnormal behaviours for all groups summed across the five initial test sessions. Bouts of epileptiform activity and limb dystonia were seen only in the low and high PPA groups. (B) The bouts occurred with equal frequency in both the low and high PPA groups and did not change in frequency during initial testing.

the intraventricular injection site and the prominence of epileptiform spiking in the hippocampal record (Fig. 2) are also consistent with a limbic origin for the kindled seizures.

Administration of PPA also resulted in the appearance of abnormal behaviours, two of which (retropulsion and limb dystonia) always occurred coincident with epileptiform events in the caudate nucleus. Limb dystonia, at times alternating with retropulsion, occurred coincident with short, distinct bouts of epileptiform spiking in the caudate. The fact that the caudate nucleus supports electrical kindling of seizures [145] raises the possibility that the caudate spiking and abnormal behaviours seen in the present study might have resulted from direct caudate nucleus kindling by PPA. However, this seems unlikely because kindling of the caudate produces limbic-type convulsive behaviour [135], at times with particular exaggeration of generalized stage 4 convulsions characterized by immediate loss of postural equilibrium followed by falling on the side, bilateral forelimb clonus, hindlimb tonic extension, and opisthotonus [145]. The abnormal behaviours seen in the present study did not resemble the severe limbic-type convulsions reported for caudate kindling by Saucier and Corcoran [145]. The additional fact that caudate kindling proceeds in a progressive manner, requiring approximately 7–17 after discharges depending on the region of caudate that is stimulated, further distinguishes caudate kindling from the abnormal behavioural responses triggered by PPA in the present study, which frequently occurred before any kindled seizure manifestations were seen.

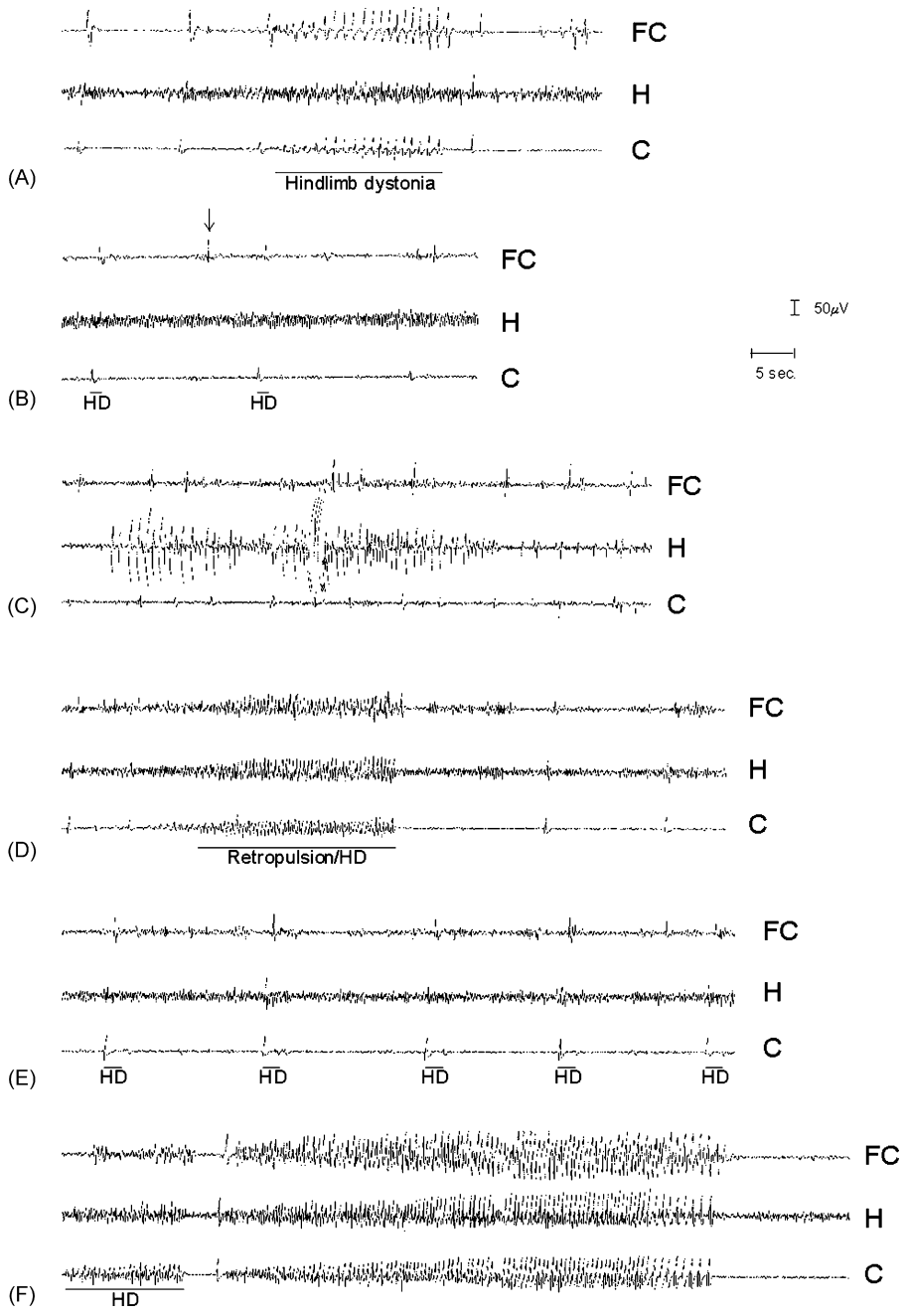

Fig. 7. Representative electrographic seizure records from Rat AUS64 in the high PPA group. (A) Session 2, short bout of epileptiform spiking accompanied by contralateral hindlimb dystonia coincident with spiking (event marker). Note spiking in frontal cortex and caudate but not dorsal hippocampus. (B) Session 3, single epileptiform spikes in caudate and frontal cortex but not hippocampus. Only the caudate spikes were accompanied by brief contralateral hindlimb dystonia (event markers), which led the frontal cortex spikes by approximately 500 ms. A prominent frontal cortex spike (arrow) is not accompanied by a spike in the caudate or by limb dystonia. (C) Session 5, bout of spiking in dorsal hippocampus not accompanied by corresponding bouts of spiking in frontal cortex or caudate or by limb dystonia. (D) Session 5, single epileptiform spikes occur first in the caudate and lead spikes in the other traces. These are followed by a short bout of epileptiform spiking that is accompanied by brief retropulsion, followed immediately by contralateral hindlimb dystonia, which ends coincident with the end of the bout of spiking (event marker). (E) Session 5 at approximately 1 min after the records in D, single epileptiform spikes in all three traces, with the caudate spikes leading the spikes in the other traces. Each spike is accompanied by brief contralateral hindlimb adduction and immediate dystonia (event markers). (F) Session 5 at approximately 6 min after the records in E, short bout of epileptiform spiking with hindlimb dystonia beginning coincident with caudate spiking (event marker), with frontal cortex and hippocampal spiking beginning after onset of caudate spiking and hindlimb dystonia. This is followed 2 s later by the beginning of the first sustained kindled seizure displayed by this rat (duration = 35 s), which was accompanied by the first conventional kindled convulsion (stage 2), which did not include limb dystonia. C: caudate; FC: frontal cortex; H: dorsal hippocampus; HD: hindlimb dystonia. Amplitude calibration: 50 μv; time marker: 5 s.

Taken together these findings suggest that the abnormal behaviours triggered by PPA administration did not depend on, and may have been unrelated to, mechanisms underlying the kindling of limbic-type seizures by PPA and the similar short chain fatty acid acetate. In particular seven of seven low PPA rats displayed abnormal behaviours prior to the first occurrence of kindled limbic-type convulsive behaviour; and these abnormal behaviours were distinct in form and did not resemble kindled limbic-type convulsions. As well, abnormal behaviours occurred coincident with single epileptiform spikes or distinct bouts of spiking

in caudate and did not require frontal cortex or hippocampal epileptiform activity to occur, whereas kindled limbic-type convulsions occurred coincident with hippocampal seizure activity and did not require caudate epileptiform activity to occur. Kindled seizures also progressively developed in a dose-related manner across test sessions, whereas abnormal behaviours associated with single epileptiform spikes or bouts of spiking in caudate did not occur in a dose-related manner and were stable across sessions. Finally, there was rapid transfer of PPA limbic type kindling to PTZ but few bouts of spiking with dystonia in response to PTZ during crossover in the same rats.

PPA possesses a number of diverse neuropharmacological properties which could be involved in the immediate electrophysiological effects as well as the longer term phenomena, such as a kindling effect including PTZ sensitivity observed in the present study. PPA is known to inhibit Na+, K+-ATPase [183], and increase NMDA receptor sensitivity [57]. Both of these properties could enhance neural depolarization, and increase glutamatergic transmission and provide plausible mechanisms for neural hyperexitability and the observed epileptiform activity.

Elevations in intraneuronal calcium levels via some second messenger systems are known to play a major role in neuroplasticity and kindling [23]. A key effect of PPA involves promoting calcium release from intracellular stores [110]. PPA is also known to elevate nitric oxide levels [173], and nitric oxide is known to play a major role in cortical, hippocampal and striatal neurotransmission, with suggested involvement in seizure and movement disorder [54]. Production of nitric oxide from activated microglia plays a major role in experimentally induced seizure and neuroinflammation [156]. These previous findings may help explain the enhanced sensitivity of PPA treated groups to PTZ-induced seizures, which requires nitric oxide for its convulsant effect [79,82].

In the present study only physiologically buffered (pH 7.5) PPA and acetate produced behavioural and electrophysiological responses, while 1-propanol, the non-acidic alcohol analogue was completely devoid of significant behavioural and electrophysiological effects. The effects common to both PPA and acetate may involve some pH or monocarboxylate-dependent process. Both PPA and acetate are weak organic acids that exist in both aqueous and lipid soluble states, the latter permitting them to directly enter the CNS as well as neurons and glia. *In vitro* studies, using organic acids including PPA at 2.5–20 mM levels, have found that cells, including those in the CNS, are capable of intracellularly concentrating these compounds and inducing reversible intracellular acidosis, particularly with minor reductions in extracellular pH [17,86,130,143,158].

Interestingly, cerebrovascular endothelium, neurons and glia possess specific monocarboxylate receptors which play an active role in the uptake of short-chain fatty acids including PPA [101]. Acetate is preferentially taken up by glia where it is rapidly metabolized via acetyl CoA and the TCA cycle into glutamine and carbon dioxide [176]. PPA metabolism in the CNS is poorly understood, but is thought to involve propionyl CoA carboxlyase, which is involved in the breakdown of PPA in the liver [19]. Elevated levels of intracellular PPA are known to increase propionyl CoA and deplete cytosolic carnitine stores, leading to increases in all short chain fatty acids, and presumably to additional reductions in intracellular pH [19,20]. Rapid metabolism of acetate by glia may explain the more rapid, but short term seizure effects of acetate in our study compared to the longer seizure durations in the PPA groups, possibly due to increased short chain fatty acid levels.

The effect of intracellular pH reductions in the CNS is complex and includes increased release of glutamate, dopamine, norepinepherine, and serotonin at cortical and subcortical levels, all of which are capable of eliciting movements such as turning, dystonia and hyperactivity [34,63,139,150,154]. Interestingly, retropulsive and dystonic movements can be produced in rodents by treatment with the propionyl derivatives 3′3′-iminodipropionitrile and 3-nitroprioprionic acid, the latter serving as a model for human Huntington's disease [109]. Thus, intracellular pH reduction, including that conceivably produced by derivatives of PPA, provides a plausible mechanism for the behaviours observed in our current and previous studies [75].

One effect of PPA known from *in vitro* studies, including those with CNS tissue, is its ability to reversibly reduce electrotonic coupling via the closure of gap junctions, presumably by inducing intracellular acidification [67,143]. Gap junctional communication plays an important role in neurotransmission in anatomical areas implicated in seizure and movement disorder, including the basal ganglia, deep cerebellar nuclei, prefrontal cortex, nucleus accumbens and the hippocampal formation [119,120]. This is thought to occur through the modulation of groups of neurons into discrete gap junction linked clusters [93]. Furthermore closure of glial gap junctions by PPA could lead to neuronal hyperexitablilty by impaired glial spatial buffering of cytosolic potassium or glutamate [2]. Thus, the altered neural excitablility of neocortical, hippocampal and striatal neuronal groups in PPA treated rats would be consistent with the closure of neural or glial gap junctions by PPA [67].

In summary, the elicitation of consistent repetitive behaviours, coupled with specific electrographic changes by PPA at the cortical and sub cortical levels is consistent with the expectations of an animal model for autism. These observations bear some resemblance to the idiosyncratic bouts of behaviours noted with autism, grouping it with the movement disorders [125], as well as its co-existence with seizure disorders [15]. The observation of caudate spikes which occur with specific dystonic behaviours in this experiment raise the possibility that similar effects may occur in human autism which would escape detection by traditional scalp electrodes, and would further implicate basal ganglial structures in the pathophysiology of this disorder [153].

4. Experiment 2—Effects of daily PPA administration on locomotor activity and oxidative stress markers

Based on the results of the initial dose-response experiment, a second experiment was completed using only the high dose of PPA to study the effects of daily PPA administrations on locomotor activity and biochemical markers of oxidative stress.

4.1. Treatment groups

Twenty-four rats were randomly assigned to the following two groups: high PPA (4.0 μl of a 0.26 M solution, $n = 12$); and PBS control (4.0 μl PBS, $n = 12$) and received ICV infusions twice daily for seven consecutive days. PPA was dissolved in PBS vehicle and buffered to pH 7.5 using HCl or NaOH. Following behavioural testing, brain tissue from this group of animals was extracted and assessed for biochemical markers of oxidative stress (see procedures below). For the biochemical analyses, all rats in the PPA group were utilized ($n = 12$) whereas only a subset of PBS ($n = 6$) animals were used.

4.2. Automated assessment of locomotor activity

Locomotor activity was measured using eight VersaMax Animal Activity Monitors (Model NVMA16TT/W, Accuscan Instruments Inc., Columbus, OH). Each monitor consisted of a 40 cm × 40 cm × 30.5 cm Plexiglas open field covered by a Plexiglas lid with air holes. Sets of infrared beams for horizontal activity measurement surrounded each open field and each beam was located every 2.54 cm for a total of 16 beams on each of four sides. Each set of infrared beams, used to measure activity, was located 4.5 cm above the floor of the open-field [121]. Light levels at the floor level of each open-field were approximately 900 lux during the light period of the light:dark cycle.

A VersaMax Analyser (Accuscan Model VSA-16, Columbus, OH) processed and relayed data from each automated open-field to a computer located in a room adjacent to the testing room. The main variable recorded by the automated open fields was total distance (TD) travelled (cm) by each rat.

Prior to all treatment sessions, untreated cannulated rats were habituated to the apparatus for two 30 min sessions. A third session of locomotor activity was recorded to establish baseline activity levels of untreated rats. Both the habituation and baseline activity recordings were completed at times that coincided with future treatment sessions.

Rats received with twice-daily ICV infusions for 7 days following the procedure outlined in Section 2.3. Infusions were completed daily at 09:00 and 13:00 h during the light period and locomotor activity was recorded on each of seven treatment days (i.e., D1–D7) for 30 min immediately following the infusion at 13:00 h. Locomotor activity was recorded for 30 min based on pilot work that showed that the majority of behavioural effects of ICV PPA treatment occurred within the first 30 min following infusion. Furthermore, the half life of PPA is known to be between 18 and 57 min when administered to rats which supports the use of the 30 min recording session for an initial investigation [25].

4.3. Biochemistry

Following the behavioural component of the experiment, brains of the PPA and PBS rats were homogenized for biochemical assays using commercial kits. Biomarkers of lipid and protein oxidation, glutathione (GSH) and the activity of enzymes involved in glutathione metabolism (glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione *S*-transferase (GST)) were studied in whole brain homogenates. Alterations in the activities of these enzymes are suggestive of reduced cellular defense and are considered to be surrogate markers of increased oxidative stress [80].

4.3.1. Tissue preparation

At approximately 09:00 h on day 8, rats were euthanized by decapitation without anesthesia, and the brain was rapidly excised, washed with ice cold PBS for blood elimination, weighed and kept on ice until homogenization. Each brain was homogenized in 10 volumes (1:10, w/v) of PBS (pH 7.4) and centrifuged at 800 × *g* for 10 min at 4 °C to discard nuclei and cell debris. The supernatant was separated and stored at −80 °C until needed for biochemical analyses and the pellet was discarded.

4.3.2. Oxidative stress marker assays

Lipid peroxidation was determined by measuring the amounts of the secondary products malondialdehyde (MDA) and 4-hydroxyalkenals (HAE), using a commercial kit (LPO-586; Calbiochem (R), La Jolla, CA), according to the manufacturer's instructions. In this assay, stable chromophore production after incubation for 40 min at 45 °C is measured at 586 nm using a Multiskan® Spectrum microplate spectrophotometer from Thermo Labsystems. Lipid peroxidation was expressed as the normalized content of MDA + 4-HNE. Values were expressed in μmol/mg of protein.

Protein carbonyl concentration was determined using the Protein Carbonyl Assay kit from Cayman Chemical®, based on the reaction between protein carbonyls and 2,4-dinitrophenylhydrazine (DNPH) to form the corresponding hydrazone [94]. Samples were incubated for 1 h with 10 mM DNPH in 2.5 M HCl. For each sample, a blank without DNPH was included. After precipitation with trichloroacetic acid, the pellet was washed three times with ethanol/ethyl acetate (1:1, v/v) and resuspended in guanidine hydrochloride. The carbonyl content was calculated from the absorbance of the protein-2,4-dinitrophenylhydrazone derivative at 370 nm using a molar extinction coefficient for dinitrophenylhydrazine at of 0.022 $\mu M^{-1}\,cm^{-1}$. Values were expressed in nmol of carbonyl derivatives per mg of protein.

4.3.3. Glutathione (GSH) system assays

Glutathione system assays were completed using available commercial kits from Cayman Chemical®. All absorbance measurements were performed in a Multiskan® Spectrum microplate spectrophotometer from Thermo Labsystems.

Glutathione peroxidase (GPx) activity was assayed based on the procedure described by Paglia and Valentine [124] using cumene hydroperoxide as substrate. The reaction was followed for 3 min at 340 nm and contribution of spontaneous NADPH oxidation was always subtracted from the overall reaction rate. GPx specific activity was expressed as U per mg of protein, where 1 U is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH per min. The molar extinction coefficient for NADPH at 340 nm is 0.00622 $\mu M^{-1}\,cm^{-1}$.

Glutathione reductase (GR) activity was determined according to Carlberg and Mannervik [36]. The oxidation of NADPH was followed for 3 min at 340 nm and the activity of GR was calculated using a molar extinction coefficient of 0.00622 $\mu M^{-1}\,cm^{-1}$. Non-enzymatic NADPH oxidation was subtracted from the overall rate. GR activity was expressed as U per mg of protein, where one U is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH per min on the basis of total protein content.

Glutathione *S*-transferase (GST) was assessed by the method of Habig et al. [71] which measures the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced GSH. The conjugation is accompanied by an increase in absorbance at 340 nm and is directly proportional to the GST activity in the sample. GST specific activity was expressed as U per mg of protein, where one unit of enzyme conjugates 1.0 nmol of CDNB with GSH per min. The molar extinction coefficient for CDNB at 340 nm is 0.0096 $\mu M^{-1}\,cm^{-1}$.

Total GSH concentrations were detected by the GSH disulphide reductase 5,5′-dithiobis (2-nitrobenzoic acid) recycling method using the procedure described in the GSH assay kit from Cayman Chemicals®. An end point method was used to calculate and determine the sample GSH concentration according to the instructions provided by the supplier. All GSH concentration values were normalized to the protein concentration within each sample. Results were expressed as μmol of GSH per mg of protein. Protein concentration was determined by the method of Lowry et al. [96] using analytical grade bovine serum albumin to establish a standard curve.

4.4. Statistical analyses

Locomotor activity data were analyzed using one and mixed design analysis of variance (ANOVA) to assess group differences (i.e., PPA and PBS) in activity levels during the baseline (BL) session as well as across each of the seven test days (i.e., D1–D7). Means of the PPA and PBS treated animals were compared by *t*-test for the biochemical data. Hypothesis tests were completed using $\alpha = 0.05$ as the criterion for significant effects. All statistical tests were calculated using SPSS 13.0 for Windows.

4.5. Results and discussion

4.5.1. Effects of PPA treatment on locomotor activity

Total distance (TD) traveled during the 30 min period is shown in Fig. 8. There was significant main effect of treatment (i.e., PPA or PBS) group ($F(1,22) = 19.94$, $p < .001$) for TD. Additional analyses revealed that PPA treated rats exhibited increased total distance (TD) traveled on D1–D6 ($F(1,22) = 4.87$; 5.16; 19.66; 4.72; 6.04; 12.93; for D1–D6, respectively, $p < .05$ or better for each day) compared to PBS treated rats however, no difference was found on D7. No significant differences occurred between PPA and PBS animals during the treatment-free baseline (BL) sessions suggesting that these samples of rats showed no inherent differences in locomotor activity prior to ICV infusions.

PPA infused rats displayed greater amounts of TD compared to controls however, this response diminished near the end of the infusion series. The increased locomotor activity found in response to PPA is consistent with the observations in experiment 1, and the expectations of an animal model of autism. The fact that the repeated infusions were well tolerated and that the rats remained healthy and active throughout the testing was encouraging for the use of PPA in an animal model. The duration of observable behaviours is also consistent with the known half life of PPA of 18.0–57.0 min when administered to rats [25].

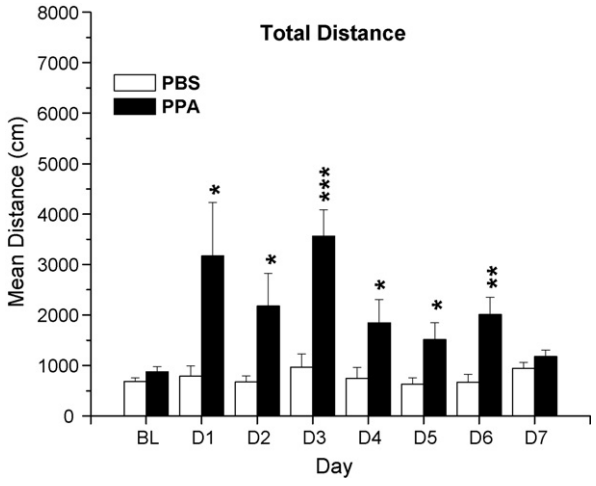

Fig. 8. Group mean (±S.E.M.) total distance traveled in rats given 4 μl intraventricular infusions of high dose PPA or PBS twice daily for 7 days ($n = 12$ per group). D1–D7 represent consecutive treatment days. PBS: phosphate buffered saline; PPA high: high dose PPA; BL: baseline session with no infusions. For all experiments $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$.

4.5.2. Effects of PPA on oxidative stress makers and GSH metabolism

Lipid peroxidation refers to the oxidative deterioration of lipids containing any number of carbon–carbon double bonds. Malonaldehyde (MDA) and 4-hydroxy-2,3-nonenal (4-HNE) are the most well studied aldehydes produced by lipid oxidation. Both can form adducts with free amino acids and proteins. In addition, MDA can introduce cross-links in proteins. Many of the reactions mediated by reactive oxygen species (ROS) conclude in the introduction of carbonyl groups into proteins. Protein carbonyls are the most commonly measured products of protein oxidation in biological samples. These moieties derive from a variety of oxidative mechanisms including (i) fragmentation, (ii) amine oxidation, (iii) metal catalyzed oxidation of specific protein amino acid side chains (Hys, Arg, Lys, Thr and Pro), and (iv) addition of unsaturated aldehydes such as 4-HNE or MDA to Lys amino groups, Cys sulfhydryl groups, and Hys imidazole groups [94].

Increased oxidative damage, putatively via neuroinflammation, mitochondrial dysfunction, and impaired GSH metabolism, may be an important aspect of autism [40]. Furthermore, biochemical processes common to those observed in oxidative stress have been proposed as mechanisms of synaptic plasticity [100]. We hypothesized that similar processes might occur in animals given intraventricular infusions of PPA. We found that PPA treatment induced a significant increase in lipid and protein oxidation, suggestive of increased oxidative stress ($t(16) = -3.09$ and -3.67, respectively, $p < .01$, for both; see Fig. 9).

Fig. 10 summarizes the effects on the GSH system. PPA treated animals showed decreased total GSH ($p < .01$) and GPx ($p < .05$), in brain homogenates of PPA treated rats indicating increased oxidative stress. GR activity was relatively stable regardless of treatment, whereas the activity of GST was increased ($p < .05$), suggesting that GSH was perhaps being used for the removal of PPA or related catabolites or, alternatively, that the production of GSH was impaired. These findings are interesting as glutathione plays a major role in cellular antioxidant defense, methylation pathways and in the integrity of the blood brain barrier. It is also a major detoxifier of a broad range of xenobiotics and metals, some of which have been suggested to be relevant as risk factors for autism [80] (see [106] for review). Reductions in brain glutathione have been found in conditions such as experimental methylmalonic acidemia [147], 2-chloropropionic acid administration [182], experimental hyperphenylalaninemia [88] and picrotoxin induced seizures [137].

Possible mechanisms mediating the GSH decrease include increased oxidation, release from the mitochondria and/or decreased import from the cytosol. However, we used a commercial kit that recycles GSSG to GSH, so the decreases observed are due to loss of GSH, possibly by conjugation in xenobiotics metabolism. Furthermore, GST was also increased suggesting that the decreased concentration of total GSH induced by PPA is likely due to GSH consumption by its conjugation with xenobiotics. This decline in GSH could render cells more susceptible to oxidative stress, which might account for the increased protein and lipid oxidation detected in the brain of PPA treated rats. However, the decreased concentration of total GSH may also be related to a reduction in ATP-dependent synthesis. The reductions in GSH could be due to the fact

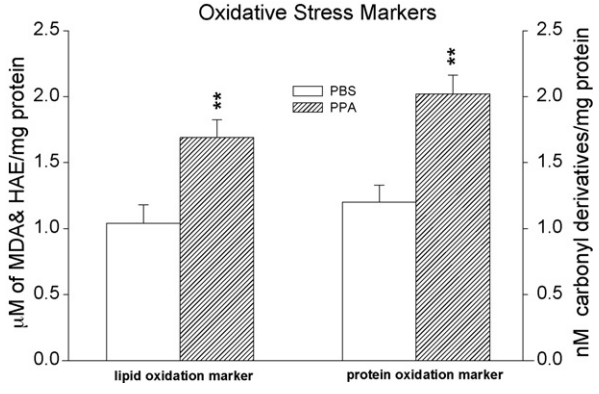

Fig. 9. Group mean (±S.E.M.) levels of lipid (left *Y*-axis) and protein (right *Y*-axis) oxidation produced after PPA or PBS treatment twice daily for seven consecutive days. $^{*}p < .05$; $^{**}p < 0.01$. MDA: malonaldehyde; HNE: 4-hydroxy-2,3-nonenal.

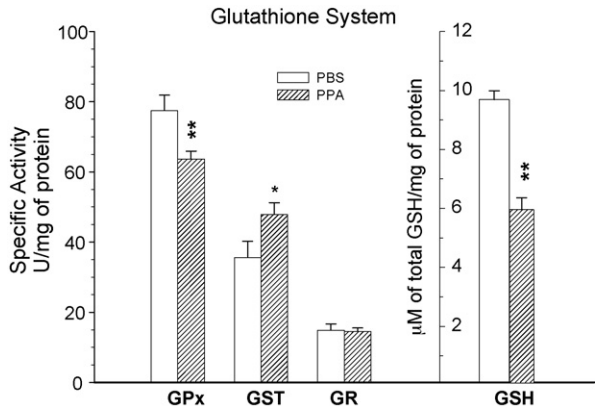

Fig. 10. Group means (±S.E.M.) of specific activity in PPA and PBS treated rats. PPA treated showed decreased total GSH. GPx was significantly decreased in brain homogenates of treated rats indicative of increased oxidative stress. Glutathione reductase activity was not different between the two groups whereas the activity of glutathione *S*-transferase increased in the PPA treated rats. $^{*}p < .05$; $^{**}p < .01$. GSH: total glutathione; GPx: glutathione peroxidase; GR: glutathione reductase; GST: glutathione *S*-transferase.

that the synthesis of GSH was impaired by PPA. However, because this decline was associated with increased GST activity these results are more suggestive of GSH being involved in the metabolic clearance of PPA or related metabolites (Fig. 11).

In summary, whether the observed reductions in GSH metabolism in our study were the result of a general impairment in GSH synthesis or due to possible sequestration of GSH by PPA or related metabolites is unclear. Whether increased oxidative stress observed in our study leads to mitochondrial failure, or alternatively, mitochondrial failure induced by PPA leads to oxidative stress is a subject of further study. Nonetheless, the observed decline in GSH concentration may render CNS cells more susceptible to oxidative stress [106], which could account for the increased protein and lipid oxidation detected in the brain of PPA treated rats, and possibly human autism.

5. Experiment 3—Effect of daily PPA administration on neuropathology

Based on the results of the first two experiments, the neuropathological effects of PPA were examined using the high dose of PPA and an infusion schedule that was extended to 13 days given that rats tolerated the 7 days administration schedule in experiment 2.

5.1. Treatment groups

Twelve rats were randomly assigned to the following two groups: high PPA (4.0 μl of a 0.26 M solution, $n = 6$); and PBS control (4.0 μl PBS, $n = 6$) and received ICV infusions twice daily for 13 consecutive days. PPA was dissolved in PBS vehicle and buffered to pH 7.5 using HCl or NaOH. Brain tissue from this group of animals was extracted and assessed for neuropathological markers.

5.2. Immunohistochemistry

5.2.1. Tissue preparation—overview

Following D13, animals were deeply anesthetized with sodium pentobarbital (270 mg/ml) and transcardially perfused with ice cold PBS (0.1 M) followed by 4% paraformaldehyde in PBS. Brains were removed and placed in 4% paraformaldehyde solution and stored at 4 °C for 24 h. Following the fixation period, brains were placed in an 18% sucrose solution prior to paraffin embedding. Selected coronal blocks from various brain regions (frontal cortex/striatum, dorsal hippocampus, midbrain and cerebellum/brainstem), were dehydrated and defatted by increasing concentrations of ethanol/xylenes and embedded in paraffin wax for permanent storage.

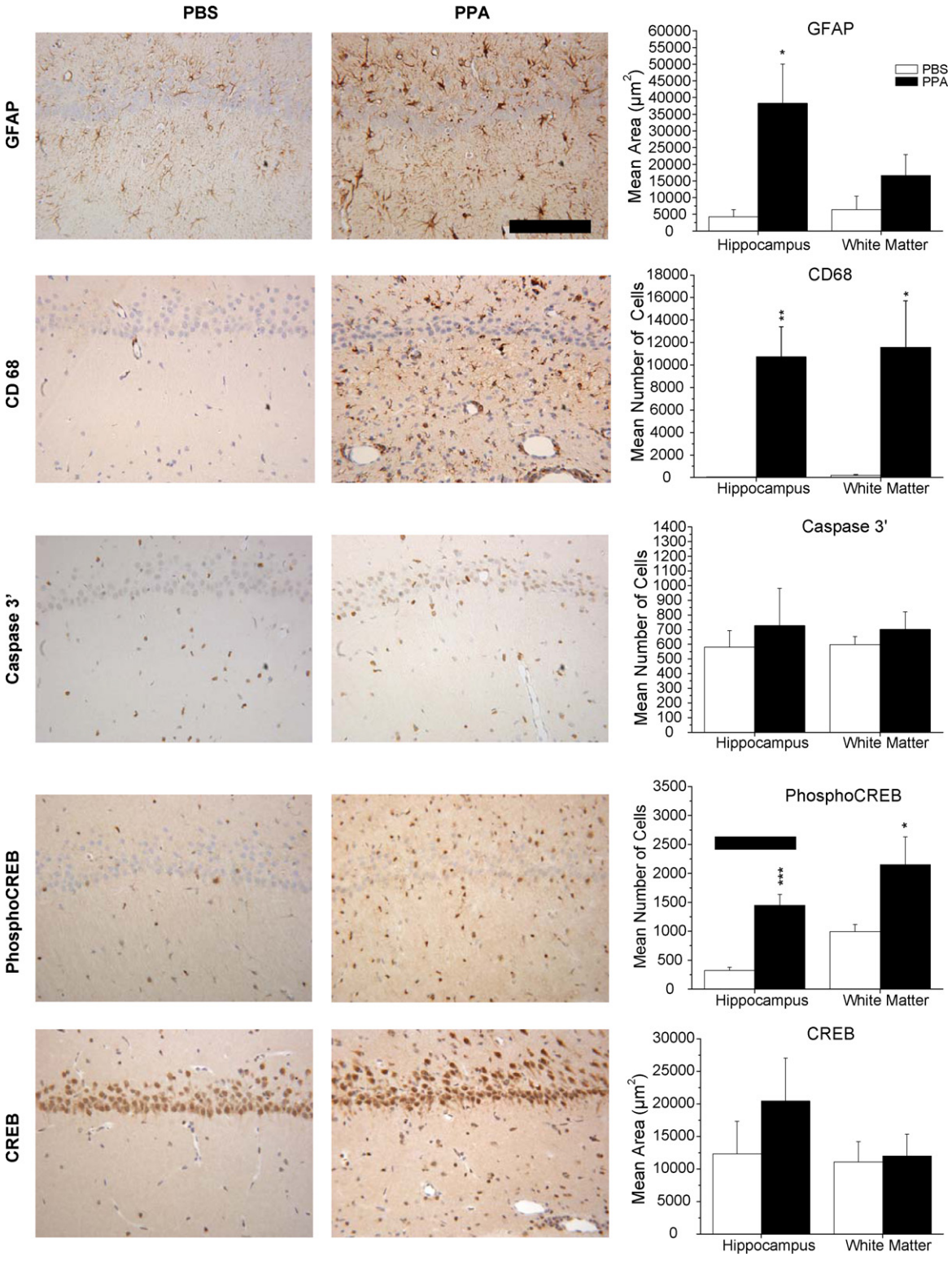

Fig. 11. Representative photomicrographs of dorsal hippocampus (CA2 region) of rats after receiving intraventricular infusions of either PBS or PPA twice daily for 13 consecutive days. Bar graphs represent group means (±S.E.M.) for each antibody in both hippoacampus and white matter of the external capsule. $^{*}p < .05$, $^{**}p < .01$, and $^{***}p < .001$ (original magnification 250×, scale bar represents 100 μm).

5.2.2. Immunohistochemistry procedures

Using a Leica microtome (model RM2125) serial 4 μm sections were obtained through the following regions: (1) right lateral ventricle (cannula site); (2) ipsilateral dorsal hippocampus, including adjacent white matter of the external capsule. These anatomical regions were chosen for preliminary analyses based on: (1) the close proximity to the intraventricular administration site of PPA or PBS vehicle; (2) the largely known cytoarchitectonics of the hippocampus for reliable neuronal quantification of possible PPA induced neuroplastic changes; (3) the known role of the hippocampus in experimental kindling, human seizure disorder and autism; (4) for analysis of

oligodendroglial changes in white matter of the external capsule, also implicated in autism.

The following antibodies were used: (1) anti-glial fibrillary acidic protein (GFAP) (1:500, polyclonal, DakoCytomation, Glostrup, Denmark) a marker for reactive astrogliosis, found elevated in human autism neuropathology studies [171] and in cerebrospinal fluid from autism patients [144]; (2) anti-rat CD68 antigen (1:200, monoclonal, Serotec, Oxford, UK) a marker for activated microglia, found elevated in human autism brain [171] and epilepsy [11]; (3) anti-cleaved caspase 3′ (1:100, polyclonal, Cell Signaling Technology, Danvers, MA), a marker for apoptotic cytotoxicity [90]; (4) anti-CREB (1:2000, rabbit monoclonal Cell Signaling Technology, Danvers, MA); (5) anti-phosphoCREB (1:5000, monoclonal, phosphorylation site serine 133, Cell Signaling Technology, Danvers, MA), test markers for neuroplasticity, calcium, G-protein and cyclic nucleotide dependent gene induction each of which is implicated as a PPA mediated effect [37].

Tissue sections were mounted on glass slides (SurgiPath, Canada) and dried overnight at 37 °C. Sections were deparaffinized and rehydrated using standard immunohistochemical procedures for antigen recovery [152]. Endogenous peroxidase activity was blocked using a 3% hydrogen peroxide in distilled water solution for 5 min. For antigen recovery, sections were immersed in boiling 0.21% citric acid buffer (pH 6.0) for 30 min in a 1250 W microwave oven. Slides were counterstained with Gill haematoxylin (EMD Biosciences) and rinsed with PBS for 5 min. A 10% normal horse serum in PBS solution was applied for 5 min followed by the primary antibodies for 1 h at room temperature. Following the incubation period, sections were washed with PBS and secondary antibodies, either biotinylated anti-mouse IgG (Vector Laboratories, Burlingame, CA, BA2000) or biotinylated anti-rabbit (Vector Laboratories, Burlingame CA, BA1000) for 30 min. Tissues were again washed with PBS and stained using the avidin–biotin complex (Vectastain Elite ABC, Vector Laboratories, Burlingame, CA, PK6100) for 30 min at room temperature. Following incubation, slides were washed with PBS and 3,3-diaminobenzidine DAB chromagen (Sigma, D8001) was applied for 5 min. After final rinsing, slides were dehydrated, cleared and coverslipped.

5.2.3. Immunohistochemistry quantification

Using a standard light microscope, eight non-overlapping digital photomicrographs (area = 160,000 μm^2) spanning the pyramidal cell layer of the hippocampus (CA1–CA4 *stratum oriens* to *stratum radiatum*) were captured at 250× magnification. From the same section of tissue, an additional seven digital images (area = 160,000 μm^2) of the white matter of the external capsule, dorsally adjacent to the hippocampus, were also captured sequentially starting at the corpus callosum and ending at the lateral ventricle. A total of 15 digital photomicrographs from a random subset of animals in each treatment group (PPA $n = 6$; PBS $n = 6$) were captured under fixed microscope illumination settings and exposure times to ensure consistent image quality across all pictures.

To quantify immunoreactivity, a standard set of color recognition criteria were created for each antibody to ensure that only DAB labelled immunopositive cells were recognized by the software. Standard color criteria were created for each individual antibody to counter the effects of variance in the intensity DAB labelling. Data from images were summed on a per-region basis to yield totals for both the hippocampus (i.e., eight images summed) and white matter (i.e., seven images summed) [122].

Two analysis methods were used for quantification of immunoreactivity. Due the diffuse nature of GFAP and CREB staining, analyses were completed by using the 'area stained' function within ImagePro Plus 5.1 software which sums the immunopositive area within a digital image to provide a total immunopositive area per picture (μm^2). For CD68, caspase 3′, phosphoCREB, each displayed immunoreactivity that was restricted within discrete cell membranes, hence these antibodies were quantified using the 'cell count' function which counted immunopositive cells only.

Finally, the cytotoxicity of PPA was also examined via direct visual cell counts of hematoxylin stained neuron cell bodies (pyramidal cells) to determine potential cell losses in the hippocampus only. Counts were completed using the images of GFAP stained sections counting non-GFAP immunopositive cells. Cell bodies were counted by a trained scorer that was blinded to the treatment. Cell bodies were counted only if they displayed an intact cell membrane and possessed a visible nucleus.

5.3. Statistical analyses

Means of the PPA and PBS treated animals were compared by *t*-test for each antibody. Hypothesis tests were completed using $\alpha = 0.05$ as the criterion for significant effects. All statistical tests were calculated using SPSS 13.0 for Windows.

5.4. Results and discussion

One PPA treated rat was eliminated from analysis due to incomplete tissue fixation leaving a total of five rats in the PPA group which was compared to six PBS treated rats. Brain tissue of PPA treated animals showed a significant increase in GFAP immunoreactivity compared to PBS controls that was suggestive of reactive astrogliosis. This finding was most striking in the hippocampus ($t(9) = -3.13$, $p < .05$), but a non-significant trend was also apparent in the white matter. Similar findings occurred for the microglia marker CD68, as PPA treated animals evidenced a greater number of activated microglia in both the hippocampus ($t(9) = -4.44$, $p < .01$) and white matter ($t(9) = -3.07$, $p < .05$) compared to PBS treated rats suggestive of heightened immune activity. Despite these differences, PPA treatment did not cause increased amounts of apoptotic cell death (caspase 3′) or pyramidal cell loss (direct cell counts), suggesting that PPA is not directly cytotoxic at doses capable of producing behavioural effects in this experiment. PPA rats showed increased PhosphoCREB immunoreactivity possibly of a glial or endovascular origin, in the hippocampus ($t(9) = -6.32$, $p < .001$) and white matter ($t(9) = -2.55$, $p < .05$). CREB immunoreactivity appeared to be increased in the nuclear element within PPA treated animals compared to PBS controls however, differences were not statistically significant.

The neuropathological observations in the rat model have some similarity to neuropathological findings from brain tissue at autopsy of autism patients [171]. Similarities included activated microglia and reactive astrocytes in hippocampus and neocortex, along with changes in white matter. We tested for increased GFAP production as a marker of a variety of toxic insults [118] and activated microglia as a marker of neuroinflammation [8]. These cell types release cytokines such as tumor necrosis factor, and macrophage chemoattractant protein, both found elevated in autism [171]. It is important to note, however, that this neuroinflammatory response has been found in a wide number of other human neurological conditions, such as Alzheimer [151] and Parkinson disease [78], epilepsy [11], multiple sclerosis [12] and AIDS dementia complex [149]. Furthermore, these findings have been substantiated by complementary rodent models such as kainic acid induced seizures [156], experimental autoimmune encephalomyelitis [133], and bacterial lipopolysaccharide [162]. Our studies are interesting as PPA induced neurotoxicity measured by direct cell counts, and caspase 3′ immunoreactivity for apoptotic effects were not found. At this stage, we cannot rule out neurotoxicity of sub populations of cells, neuronal sub lethal effects, or possible neuronal toxicity in other brain areas following repeated administrations of PPA.

The initiation of PPA induced behaviours within minutes (as observed in experiments 1 and 2), even following the first infusion, suggests the involvement of rapid biochemical mechanisms such as signal transduction or pH, but the induction of longer term mechanisms such as protein synthesis and gene induction, cannot be ruled out. Suggestive of the latter is the increased immunoreactivity of the phosphorylated version of the cAMP response element binding protein (pCREB), but not the non phosphorylated version of CREB, in putatively glial and endothelial elements of hippocampus and white matter observed in PPA treated rats. We chose CREB and pCREB as an initial test marker, as it is expressed in all cells in the CNS, and is important in learning and memory and plays a key role in the transmission of membrane events into widespread alterations in gene expression (see [37] for review). In mice, increased CREB phosphorylation has been observed with many PPA-dependent events including dopamine and NMDA receptor activation [115], experimental seizure [115], and neuroinflammation [35]. Thus PPA, via CREB-dependent pathways may be in a position to induce widespread gene expression thereby providing a plausible mechanism by which this fatty acid may produce long term effects.

It should be noted that the behavioural effects observed in experiment 2 showed a return to baseline levels suggesting some compensatory processes may be involved. We cannot ascribe exact mechanisms at this time for this preliminary study, but plausible pathways could include increased synthesis of PPA

metabolizing enzymes such as propionyl CoA decarboxylase, induction of carbonic anhydrase as a response to acidosis [116] or induction of anti-inflammatory cytokines such as TGF-Beta or interferon-beta by the observed proliferation of microglia [74]. It is also not yet possible to determine if the neuropathological findings observed in our model are causative or compensatory to some or all of PPAs observed behavioural or electrophysiological effects [160].

6. General discussion

Typically an animal model is unlikely to replicate a human disease. The utility of such models relates to the various types of validity that can be shown to exist for specific models. Face validity, or the degree to which the model is able to capture the phenomenology of the disorder, is usually an essential first step in developing a model [180,181] but additional evidence for construct validity, i.e., a sound theoretical rationale for the model, possibly based on etiology is equally as important. Crawley [50] has provided a list of symptoms of ASD that would aid in the establishment of face validity for a rodent model, along with suggested behavioural tests for such symptoms.

In the initial development of our model of ASD we focused on four broad aspects, behavioural, brain electrographic, biochemical markers and neuropathological characteristics which would provide some face, as well as, construct validity for the model (see partial list in Crawley [50]). At a behavioural level we were interested in determining whether or not PPA would induce hyperactivity, stereotypies and repetitive behaviours, as well as abnormal motor movements. Electroencephalographic recordings allowed us to monitor for cortical and sub-cortical epileptiform activity and development of seizures along with behavioural assessment of seizures. Finally, we also looked for possible neuropathological and biochemical markers that might be consistent with those observed in humans, such as neuroinflammatory astro and microglial changes, as well as biochemical effects suggestive of increased oxidative stress or metabolic dysfunction.

These initial studies provide support for the face validity of the intraventricular PPA administration adult rat model of autism, as the behavioural and electrographic changes observed resemble those seen in the human condition. The effects of PPA in rats include hyperactivity, repetitive and abnormal motor movements, as well as the presence of seizures, both behaviourally and electrographically. The rapid induction of these behavioural and electrographic effects and their potentiation with repeated exposures, as well as the absence of such behaviours or kindled seizures in rats receiving control compounds (1-propanol and sodium acetate), suggests the involvement of PPA activated neural mechanism(s).

Evidence from human studies suggests that autism is a condition that may involve an ongoing neuroinflammatory disorder possibly resulting from an increased sensitivity to oxidative stress from a variety of environmental risk factors. The neuropathological and biochemical findings of our model support this hypothesis. Interestingly, the observed impairments in the GSH system of our model are consistent with the findings in human autism cases and would provide a plausible mechanism for increased environmental sensitivity to a number of factors. The similarities in neuropathological and biochemical changes between the animal model and human ASD cases could represent similar underlying etiological processes subsequent to central nervous system insult with PPA. Clearly much additional work needs to be done before these similarities can be taken as support for construct validity of the animal model.

It is important to note that there are no studies that have examined PPA and related metabolites in ASD. However, there is some indirect evidence suggestive of increased PPA, possibly via impaired fatty acid metabolism. A relative carnitine deficiency [62], and systemic elevations of nitric oxide metabolites [155], both known to be elevated by PPA in experimental systems, have been noted in autistic patients. The antiepileptic drug valproate, a prenatal risk factor for autism [77,179], alters both fatty acid [48] and biotin metabolism [148], and causes the depletion of carnitine stores [48] each of which could theoretically increase PPA levels. Prenatal exposures to ethanol, known to produce developmental delay [47,70], also increases PPA levels to millimolar levels [52], putatively by depleting intracellular carnitine stores [33].

Despite the lack of studies on PPA, related fatty acids and ASD, abnormalities of lipid metabolism have been postulated as a potential cause of the condition. Patient studies have noted impairments in mitochondrial beta oxidation of fatty acids [42], reductions in essential polyunsaturated fatty acids levels [170], and anecdotal reports of improvement in some patients with essential fatty acid supplementation [141].

The present studies examined the effect of intraventricular PPA in adult rats. Given the intriguing multiple effects of PPA on neurological, gastroenterological and immunological processes, together with our initial findings on behaviour, electrophysiology, neuropathology and biochemistry, the rat intraventricular PPA model may be a useful paradigm for the examination of the pathophysiology of ASD. However, autism is a neurodevelopmental disorder, with evidence of altered nervous system development, involving white matter abnormalities and cortical dysplasia. Thus, treatment with PPA and appropriate control compounds during critical times of pre- and post-natal development is an essential next step in extending the validity of this animal model.

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