Research report

Low-dose chronic prenatal alcohol exposure abolishes the pro-cognitive effects of angiotensin IV

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Abstract

Prenatal ethanol exposure (PAE) in humans results in a spectrum of disorders including deficits in learning and memory. Animal models to date have typically used high ethanol doses but have not identified the biochemical changes underlying the cognitive deficit. This study used treatment of mouse breeding harems with 5% ethanol via drinking water throughout pregnancy and lactation and explored the behavioural consequences in the progeny at 3–6 months of age using the open field test, novel object recognition test and elevated plus maze to measure anxiety and memory consolidation. The effects of angiotensin IV on behaviour of the progeny were also determined. The results indicated that PAE increased anxiety-like behaviour as determined in the open field test in male but not female progeny. In control animals, angiotensin IV enhanced memory consolidation in males, but this effect was abolished by PAE. The abolition of the pro-cognitive effect of angiotensin IV was not a consequence of increased anxiety, and there was some evidence of a long-lasting anxiolytic effect of angiotensin IV in the male PAE progeny. These results suggest that PAE may act via alteration of the actions of the brain renin-angiotensin system to impair memory consolidation, but these effects may be partially sex-dependent.

Keywords: Fetal alcohol spectrum disorder; Prenatal alcohol exposure; Learning and memory; Angiotensin IV; Anxiety

1 Introduction

In humans, prenatal alcohol exposure (PAE) can cause adverse effects on the brain of the developing foetus and can result in a wide range of neurobehavioral deficits, encompassed by the term fetal alcohol spectrum disorders (FASD) [1]. Fetal alcohol syndrome (FAS) is considered the most severe form of these disorders and is characterised by offspring displaying neurodevelopmental abnormalities of the CNS, such as small head size at birth; structural

brain abnormalities and specific neurological signs (e.g., impaired fine motor skills, neurosensory hearing loss, poor tandem gait, poor eye-hand coordination, cognitive impairment). Moreover FAS is associated with growth retardation, such as low birth weight, lack of weight gain over time, disproportional low weight to height and a characteristic pattern of facial anomalies, including short palpebral fissures, flat upper lip, flattened philtrum and flat midface [2,3]. Anxiety has also been associated with FAS and FASD, with the offspring commonly displaying depression- and anxiety-related traits [4,5]. Even low doses of alcohol are believed to be able to generate this effect [6,7].

The risk factors for FAS are unclear, but factors such as socioeconomic class, ethnicity, alcohol metabolism and pattern of drinking seem to be important [8]. There are many difficulties with the estimation of the prevalence of FASD, but conservative figures suggest that it may be approximately 2–7 cases per 1000 live births in the developed world [9,10]. Higher rates have been reported from countries with historically high prevalence of alcohol consumption [11].

Rodent models of PAE that have been used to study the pathology of FASD help to control for the confounding variables that exist in the human context [12,13] and Sulik and her group, for example, have demonstrated a clear teratogenic effect of ethanol with PAE in rats being associated with facial dysmorphology characteristic of FAS [eg 14]. Rodent models of FASD have been created with numerous ethanol administration methods, from inhalation in vapour chambers to injections (subcutaneous or intraperitoneal) and ingestion. The latter has proved popular with groups using intragastric gavage, liquid diet models or voluntary drinking paradigms [reviewed in 15 and 16]. In rats, typical oral ethanol doses used range from 3 to 16 g/kg/day, with a mode of approximately 5 g/kg/day. In mice, typical oral ethanol doses used range from 5 to 24 g/kg/day, with a mode of approximately 16 g/kg/day. These give rise to blood alcohol concentrations of 100–700 mg/100 ml [12]. In humans, blood alcohol concentrations of approximately 400 mg/100 ml are seen as potentially fatal [17], thus not all of these models are realistically reflective of the human situation. Several animal models of FASD have identified behavioural deficits associated with PAE [18] but the neurochemical aetiology of FASD remains to be resolved although studies using *in vivo* and *in vitro* models have highlighted a multitude of potential mechanisms by which PAE disrupts neurogenesis and causes cell death, for example oxidative stress, disruption of growth factors (such as BDNF) and changes in the transport and uptake of glucose [19–21]. Animal models of heavy maternal alcohol consumption and binge-drinking have revealed that PAE is associated with impaired performance in behavioural tasks that assess learning and memory and cognitive deficits [22–24].

Several groups have investigated cognition enhancing drugs and assessed their ability to reverse the deleterious effects of prenatal exposure to ethanol, for example vitamin E and the anti-dementia drug memantine [25,26]. One such cognition-enhancing drug is the peptide angiotensin IV, a component of the renin-angiotensin system that has been shown to have beneficial effects on memory acquisition and recall in rats and mice [27,28]. Both central and peripheral administration of angiotensin IV has been shown to improve novel object recognition in rats and mice [28–31]. Furthermore Pederson et al. have shown that it can counteract drug-induced impairment of learning and memory [32]. Together these studies demonstrate that exogenous administration of angiotensin IV can enhance learning and memory in healthy rats and mice and reverse memory deficits observed in animal models of amnesia. Human trials have also demonstrated that drugs known to interfere with the renin-angiotensin system, such as angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor antagonists (AIIAs), also improve learning and memory in ageing patients and young, healthy volunteers [33–35], possibly via effects on angiotensin IV [36].

The aim of this study was to explore the effects of angiotensin IV in learning and memory in a model of PAE in which mice were exposed to 5% ethanol in the drinking water throughout pregnancy and suckling. The study gives some insight into the involvement of the brain renin-angiotensin system in the aetiology of the cognitive deficits associated with FASD.

2 Materials and methods

2.1 Animal husbandry

All procedures were licenced under the UK Animals (Scientific Procedures) Act 1986 and EU directive 2010/63/EU and complied with the ARRIVE guidelines. They were approved by the University of Brighton animal welfare and ethics review board. C57BL/6J mice were maintained at 19.0 ± 1 °C, 55% humidity and fed on either a breeding diet (RM3 (E) 801002 chow, Special Diet Services) (breeding harems) or a maintenance diet (RM1 (E) 801002 chow, Special Diet Services) (offspring) *ad libitum*. The mice were maintained on a 12-h light/dark schedule, lights on 0700 h (60 Lux at cage level). Behavioural testing was conducted between 1000 and 1500 h.

2.2 Prenatal alcohol exposure (PAE)

Breeding harems were established with one male to two females; males were removed from the cages once pregnancy had been confirmed. The harems received fluid *ad libitum* with the alcohol exposure groups having 24 h access to a bottle of 5% ethanol and the control group having 24 h access to a bottle of tap-water. The volume of liquid consumed by each harem was recorded daily. Because the number of mice per cage varied as a consequence of the males being removed on confirmation of pregnancy it was assumed that the consumption was approximately equal between different mice within each cage in order to estimate individual alcohol consumption. No account was taken of litter size when estimating fluid intake. Offspring were weaned at 20 days and group-housed in same-sex, littermate cages with free access to food and tap-water. Behavioural assessments were made of the control and PAE progeny at 3–6 months of age.

2.3 Blood alcohol concentration determination

Blood alcohol concentration was determined by gas chromatography. Briefly, blood was collected by cardiac puncture post mortem from a small sample of male and female ex-breeders between 1100 h and 1300 h. Plasma samples were deproteinated with 10% trichloroacetic acid spiked with 1% ethanol and centrifuged at 9400*g* for 10 min. The supernatant was filtered before gas chromatography analysis. Standards and samples, both with an internal standard (1% propan-1-ol), were analysed on a Perkin Elmer Clarus 500 gas chromatograph with a Zebron Phase (ZB-waxplus column), equipped with a flame ionization detector. The optimal operating conditions were as follows: oven temperature 35 °C and flame ionisation detector temperature 150 °C, with the injection temperature 250 °C and the capillary temperature 250 °C; hydrogen and air pressure were set at optimal conditions of 58 psi and 60psi, respectively.

2.4 Behavioural tests

All behavioural tests were conducted within a sound-proof chamber (other than for the constant hum of a small ventilation fan). The lighting was constant at 40 Lux, slightly below that of the home cage. The experimental protocol was:

Time 0 h: 3 min exposure to open field.

Time 1 h: First 3-min training period, novel object recognition test.

Time 2 h: Second 3-min training period, novel object recognition test, followed immediately by administration of angiotensin IV or vehicle control.

Time 26 h: 3-min novel object discrimination period.

Time 27 h: 3-min elevated plus maze.

Within all experimental sessions, different treatment groups (i.e. control vs PAE; saline vs angiotensin IV) were alternated to remove any environmental/time of day confounding effects.

2.4.1 Open field test

Mice were placed inside the open field (34 × 60 cm) and allowed 3 min to explore the area before being returned to their home cage. The field was cleaned with 70% ethanol before the test session and between each animal. The behaviour was video recorded and analysed using Ethovision software to track total distance moved during each trial and the time spent with the centre-point of the animal within 5 cm from the walls of the apparatus (thigmotaxis).

2.4.2 Novel object recognition

The novel object recognition test was adapted from the protocol previously described [37]. Briefly, 1 h following the open field test the mice were returned to the same field (34 × 60 cm) into which two identical objects (plastic building blocks) were positioned at one end of the field, each 5 cm from the adjacent walls. The mice were allowed to explore the field and objects for 3 min before being returned to their home cage. The mice were again exposed to the field and familiar objects one hour later. Immediately after the second familiarisation trial the mice were treated with angiotensin IV (5 μg/kg, s.c., Bachem AG) or saline control, all at 10 ml/kg, before being returned to their home cage for 24 h.

For the final (discrimination) trial, one of the objects was replaced with a novel (different shape and colour) object, and the mice were again given 3 min to explore the field and objects. Novel objects were replaced, left and right, on an alternate basis to remove bias. Both the field and objects were cleaned with 70% ethanol between each animal during all stages of the procedure. At all stages the animal behaviour was video recorded and analysed using Ethovision software to track total distance moved during each trial and the time spent with the nose-point of the animal within 5 mm of each of the objects: the definition of object exploration as defined by [38].

2.4.3 Elevated plus maze

The elevated plus maze was used to assess anxiety in mice one hour after the novel object recognition test discrimination trial, 25 h after angiotensin IV administration. Briefly, mice were placed onto an elevated cruciform runway, with four arms (each approximately 8 cm wide and 30 cm long). Two opposite arms were enclosed by high walls (approximately 15 cm high), and the other two arms were open. The runway was elevated 1 m from the floor. Mice were placed in the centre zone of the maze and left to explore for 3 min, after which they were returned to their home cage. As with the previous tests, the maze was cleaned with 70% ethanol between each animal. At all stages the behaviour was recorded and analysed using Ethovision software to track total distance moved during each trial and the time spent in both open and closed arms.

2.5 Behavioural statistical analysis

The distribution of data were assessed for normality using the Kolmogorov-Smirnov test.

Data for fluid intake, body weight and total distance moved on the elevated plus maze were found to be normally distributed and values are expressed as group means ± standard error of the mean (sem). Comparison of group means was by Student's *t*-test where two groups were compared or by one-way or two-way analysis of variance (ANOVA), followed by post-hoc tests as appropriate, where more than two groups were compared.

Data for locomotor activity within the open field, thigmotaxis, distance moved and time spent exploring objects in the novel object recognition test and time spent exploring the open arms in the elevated plus maze were found not to be normally distributed. Data are presented as box plots which display median value, interquartile range and maxima and minima and outliers. Group data for paired and unpaired values were compared using the Mann-Witney and Wilcoxon tests respectively where two groups were compared and the Kruskal-Wallis test were more than two groups were compared.

A p value <0.05 was considered to be statistically significant.

3 Results

3.1 Alcohol intake and blood alcohol concentrations

Daily fluid consumption (calculated per animal) was recorded for two breeding harems of each treatment group over 10 weeks during which each harem produced two litters. Two-way ANOVA confirmed that there were significant changes in fluid intake over time ($p = 0.0022$) and that there was a significant difference between the two treatment groups ($p = 0.0052$, Fig. 1). The mean daily volume of fluid ingested per animal was significantly lower in the alcohol group compared to control (7.10 ± 0.70 ml v 10.04 ±0.47 ml). Fluid intake varied throughout the 10 week period, from a baseline of approximately 5 ml per day in the alcohol group at the start of the study to a maximum of 12 ml per day in late pregnancy/lactation. Only 2 of the 4 animals sampled had measurable blood ethanol concentrations between 1100 h and 1300 h. These two samples had highly disparate values of 87.5 and 0.294 mg/100 ml. The group mean blood alcohol concentration was thus 21.95 ± 21.85 mg/100 ml.

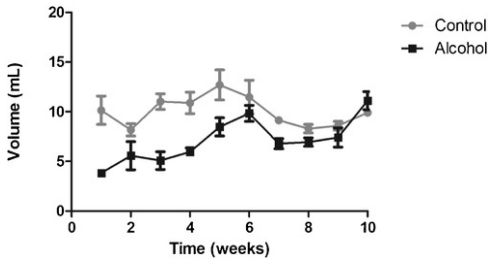

Fig. 1 Maternal drinking patterns, expressed as estimated mean volume intake per mouse, over the 10 week breeding period. Litters were delivered at approximately 4 and 9 weeks. Data shown as mean ± S.E.M of two cages of 2–3 animals per group, the alcohol group drank significantly less than the control group over the duration of the study ($p < 0.01$ 2-way ANOVA).

alt-text: Fig. 1

3.2 Progeny body weight

Progeny weight was monitored immediately before behavioural testing, for both males and females (Fig. 2). There was a significant difference between the sexes ($P < 0.0001$, 2-way ANOVA) but no significant difference in body weight between the different treatment groups for either male or female offspring, nor significant sex x treatment interaction.

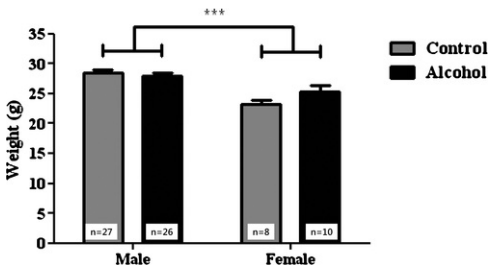

Fig. 2 Weights of control and pre-natal alcohol-exposed male and female mice at the time of behavioural experiments (3–6 months of age). No significant differences in weight were detected in either male or female progeny, although there was a significant difference in body weight between males and females. Data shown as mean ± S.E.M. of indicated sample sizes (*** $p < 0.0001$).

alt-text: Fig. 2

3.3 Open field test

There were no significant effects of PAE on locomotor activity within the open field in either male or female offspring (Fig. 3A). With respect to the percentage time the mice spent close to the walls of the open field (thigmotaxis) all mice showed a tendency to avoid the open centre-ground. In male offspring the pre-natal alcohol exposure significantly increased thigmotaxis ($p < 0.001$, Mann-Whitney test, Fig. 3B) but this was not the case for female offspring.

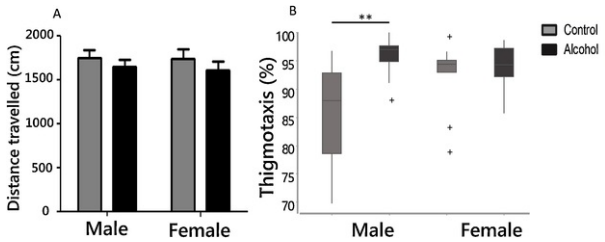

Fig. 3 Effect of pre-natal alcohol exposure on locomotor activity and thigmotaxis during the open field test in male and female progeny. (A) There were no significant effects of PAE on locomotor activity in male or female offspring. (B) In male progeny, PAE resulted in significantly greater thigmotaxis; there was no effect of pre-natal alcohol exposure in female progeny. Box plots represent median value and interquartile range, whiskers represent maxima and minima. + represents outliers. n = 36 for control males, 38 for PAE males, 12 for control females and 24 for PAE females (** $p < 0.001$, Mann-Whitney test).

alt-text: Fig. 3

3.4 Novel object recognition

Within the novel object recognition test, there was no significant effect of prenatal alcohol exposure nor angiotensin IV treatment on total distance moved within the 3 min of the test (discrimination trial) (Kruskal-Wallis test, Fig. 4A).

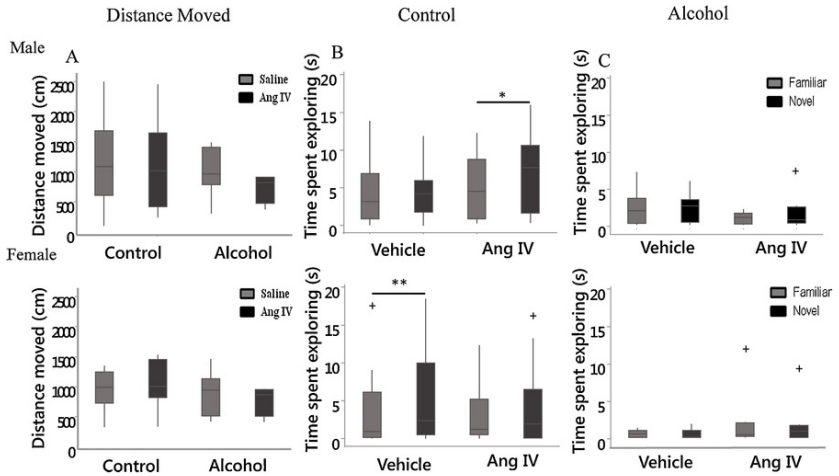

Fig. 4 The effects of pre-natal alcohol exposure and 24-h pre-treatment with angiotensin IV (5 μg/kg s.c., Ang IV) on the behaviour of male and female mice in the novel object recognition test. (A) Illustrates that neither pre-natal alcohol exposure nor 24-h pre-treatment with ang IV significantly influenced locomotor activity in the novel object recognition test in either sex progeny. (B) Illustrates that in the control groups, male mice discriminated significantly between the novel and familiar object only after ang IV pre-treatment and female progeny discriminate significantly between novel and familiar objects only after saline vehicle pre-treatment. (C) Illustrates that neither male nor female progeny exposed to pre-natal alcohol discriminated significantly between novel and familiar objects even after ang IV pre-treatment. Box plots represent median value and interquartile range, whiskers represent maxima and minima. + represents outliers. n = 16-20 for control males and females and 8 for PAE males and females (* $p < 0.05$ and ** $p < 0.001$, Mann-Whitney test).

alt-text: Fig. 4

With respect to the paired analysis of the exploration of the familiar and novel objects, the results demonstrated that the male control progeny showed no significant discrimination between novel and familiar objects following treatment with saline vehicle, but significantly greater exploration of the novel object following pre-treatment with angiotensin IV ($p = 0.049$, Wilcoxon test, Fig. 4B). In male PAE progeny, again there was no significant discrimination between novel and familiar objects by those animals pre-treated with saline vehicle, but the pre-natal ethanol exposure abolished the effects of angiotensin IV pre-treatment on object discrimination that had been seen in the non-PAE group (Fig. 4C).

In female control progeny, animals pre-treated with saline vehicle discriminated between the novel and familiar objects ($p = 0.009$, Wilcoxon test, Fig. 4B), but those pre-treated with angiotensin IV showed no significant discrimination. In female progeny exposed pre-natally to ethanol, neither the saline vehicle group nor the group that received angiotensin IV-pre-treatment showed any discrimination between novel and familiar objects (Fig. 4C).

With respect to the total time spent exploring the objects (familiar + novel objects), there was a significant effect of prenatal ethanol exposure on total exploration within the 3 min of the test (discrimination trial) with PAE reducing distance moved in both male and female offspring ($p = 0.004$ and 0.001 respectively, Kruskal-Wallis test). There was no significant main effect of pre-treatment with angiotensin IV on total exploration of objects in either male or female offspring.

3.5 Elevated plus maze

In the male progeny, the total distance moved as part of the elevated plus maze test was significantly greater in the PAE animals than the controls ($p = 0.02$ 2-way ANOVA, Fig. 5A). There was no significant influence of the angiotensin IV pre-treatment nor was there a significant interaction. In female progeny there was no significant effect of PAE nor angiotensin IV pre-treatment on total distance moved.

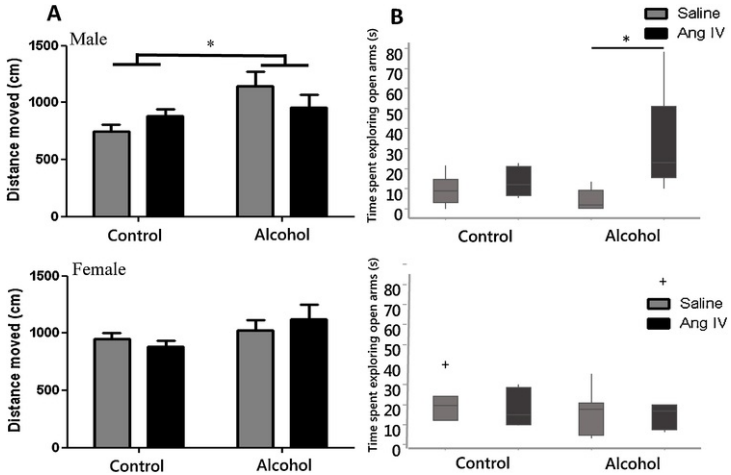

Fig. 5 The effects of pre-natal alcohol exposure and 25-h pre-treatment with angiotensin IV (5 μg/kg s.c., Ang IV) on the behaviour of male and female mice on the elevated plus maze. (A) Pre-natal exposure to alcohol caused a significant increase in distance moved in the male but not female progeny (* $p < 0.05$). (B) Pretreatment with angiotensin IV had no significant effect on the time spent on the open arms in either male or female contraol progeny. IN PAE animals, angiotensin IV significantly increased the time spent in the open arms in male progeny but not female. Box plots represent median value and interquartile range, whiskers represent maxima and minima. + represents outliers. n = 7–12 for control males and females and 7–8 for PAE males and females (* $p < 0.05$, Mann-Whitney test).

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With respect to time spent on the open arms, in male progeny there was no significant effect of angiotensin IV pre-treatment in the control animals, but angiotensin IV pre-treatment significantly increased the time on the open arms in the alcohol-treated animals ($p = 0.0014$, Mann-Whitney test, Fig. 5B).

In the female progeny, neither pre-natal alcohol exposure nor angiotensin IV pre-treatment had any significant effect on time spent on the open arms of the elevated plus maze.

4 Discussion

This study investigated the effect of low dose alcohol exposure throughout pregnancy and suckling on the behaviour of the adult offspring. The average ethanol intake per breeding female was approximately 11 g/kg/day. Other groups using single bottle models have reported average consumptions of 10–14 g/kg/day [39,40]. Taking into account the different apparent volumes of distribution for ethanol (total body water) in female humans and mice (0.63 l/kg v.

0.8 l/kg) and the greater rate of ethanol elimination in mice [41], the ethanol intake in the current study equates to a human daily ethanol intake of approximately 100 g, the equivalent of one bottle of table wine. These daily consumptions in mice have been reported as resulting in blood alcohol concentrations averaging 80–120 mg/100 ml [24,39]. Our results identified concentrations significantly lower than these, which could be explained by rodents drinking primarily during the dark phase (which in the current would be 1900–0700 h) compared to blood sampling some 5 h later. Allan et al. [39] monitored blood alcohol concentrations of B6SJL/F1 mice over a 24 h period (with lights on 0700–1900) and found that administration of 14 g/kg/day of ethanol, resulted in peak blood alcohol concentrations of 140 mg/100 ml at approximately 0100 h and minimum concentrations of 50 mg/100 ml between 0900 and 1200 h. Our blood collection was conducted at the time of lowest blood alcohol and it revealed very variable blood alcohol concentrations, although a mean of approximately 22 mg/100 ml clearly indicates that our 11 g/kg/day average-model results in lower-dose exposure to alcohol than the 14/g/kg/day. Another difference between the current study and that of Allen et al. [39] may be mouse strain: C57BL/6J versus B6SJL/FI, although the latter were derived from the former.

In humans, FASD is associated with decreased body weight and retarded growth [2]. The results of the current mouse model study failed to find any effect of the low dose PAE on offspring body weight at 3–6 months. Such results are in keeping with the work of Kaninen-Ahoha et al. [42] who used cross-fostering to show that pre-natal exposure of C57/BL6J mice to 11 g/kg/day from gestational days 1–8.5 resulted in significantly decreased body weight at age 21 days that was not a result of poor maternal behaviour, but that there was no significant effect on body weight after 5 weeks of age. In rats, however, treatment of dams throughout pregnancy and lactation with 4.5 g/kg/day resulted in significantly decreased weights in male offspring at 3 months, but no differences in female offspring [43]. These combined results suggest a species difference in the effects of PAE on body weight but support the current mouse model of PAE as being similar to those of others.

With respect to the behavioural findings, Human FASD has been associated with hyperactivity [1], and this has also been shown in one mouse model using exposure to 10% ethanol in drinking water from gestational days 0–8 using the open field test [44]. A lower dose of ethanol, 6 g/kg/day from gestational days 6–15, however has been shown to significantly decrease locomotor activity in the open field test [45]. At the exposure concentration utilised in the current study there was no evidence of hyper- nor hypo-activity of the progeny with no significant effect of pre-natal ethanol exposure on total activity in the open field test, nor the novel object recognition test in either male or female progeny.

Human, FASD has also been associated with an increase in clinically-relevant anxiety [5]. In our mouse model the open field test revealed an increase in anxiety in male PAE offspring, as determined by the extent of thigmotaxis. Female offspring did not appear to be affected in the same way but the sex difference may have been a result of a ‘ceiling’ effect; both males and females showed marked thigmotaxis, in the region of 90%, but with the control female mice showing greater thigmotaxis than the control males. This high basal value in females may have precluded identification of any additional anxiety-like behaviour of PAE using this model. Such PAE-elevated anxiety has been seen in other animal models, for example mandarin voles treated orally with 750 mg/kg ethanol daily from gestational day 14 to postnatal day 1 and Swiss albino mice treated with 6 g/kg/day ethanol from gestational days 6–15 [45,46].

The findings of the novel object recognition test need to be considered from several perspectives: Firstly, the parameters chosen (two 3-min training sessions and a recall session 24 h later) do not typically demonstrate any learning within the control mice [37]. The aim of this series of experiments was therefore to ascertain the effects of PAE on the effects of angiotensin IV. Angiotensin IV has previously been shown to enhance cognition using the above-mentioned paradigm [37]. Secondly, analysis of the results for the non-angiotensin IV-treated animals allows some consideration of the effects of PAE on exploratory and inquisitive behaviour. Finally, in the light of the fact that angiotensin IV is known to bind to the angiotensin AT_1 receptor, that AT_1 receptor stimulation has been shown to have anxiogenic effects [47] and that anxiety will confound learning and memory assessments, the results need to be considered in tandem with a timely assessment of anxiety-like behaviour. The use of the elevated plus maze one hour after the novel object recognition test recall trial was designed to fulfil this need.

The results showed that within the animals that did not receive pre-treatment with angiotensin IV (saline treated), PAE had no significant effect on total distance moved in either male or female offspring. These results indicate that PAE causes neither long lasting sedation nor motor deficits and are in-line with the findings of the open field test. Similarly, there was no significant main effect of angiotensin IV pre-treatment in either males or female offspring indicating that angiotensin IV does not have any marked stimulant nor sedative effect, nor does it induce any motor disruption.

When considering the differentiation of the familiar and novel objects, the results indicate that male mice treated with saline showed no significant discrimination. Following treatment with angiotensin IV, however, the mice differentiated significantly between the novel and familiar objects. This result replicates our earlier findings [37]. Female mice, however, behaved quite differently: those animals receiving saline vehicle demonstrated significant differentiation, a result not unique in our laboratory, but not typical. Following treatment with angiotensin IV the discrimination was lost; our previous work in male mice demonstrated loss of discrimination at higher doses of angiotensin IV [37], thus this result may represent some form of high dose effect with some other action of angiotensin IV becoming apparent.

In those males pre-natally exposed to ethanol, the pro-cognitive effects of angiotensin IV were lost, suggesting that PAE prevents the effects of angiotensin IV on memory consolidation. Importantly, however, PAE significantly decreased the total time exploring the objects in both saline- and angiotensin IV pre-treated male and female offspring. Whether this reduction in exploratory and inquisitive behaviour by PAE explains the loss of efficacy of angiotensin IV remains to be clarified, but taken with the OFT results it does suggest that the animals have similar locomotor activity but less targeted exploration, possibly a consequence of the thigmotaxis.

Results of the elevated plus maze assessment conducted shortly after the novel object recognition test are confusing. There is no evidence of increased anxiety-like behaviour, as determined by decreased time spent on the open arms of the maze, in either male or female control or PAE offspring. Furthermore, 27-h pre-treatment with angiotensin IV did not increase anxiety-like behaviour in any of the treatment groups. It can therefore be stated confidently that the observed PAE-induced deficits in novel object recognition test were not a consequence of increased anxiety-like behaviour. The elevated plus maze results do, however, indicate that PAE together with 27-h pre-treatment with angiotensin IV reduces anxiety-like behaviour in male offspring. This is against the preliminary expectation because, as described above, it was predicted that angiotensin IV, acting via AT_1 receptors would increase anxiety. The renin angiotensin system has been previously linked to anxiety, with angiotensin II having an anxiogenic effect and angiotensin receptor (AT_1) antagonists and ACE inhibitors having an anxiolytic effect [48–50]. Moreover, the AT_1 but not AT_2 receptors are thought to be involved in increased anxiety [48]. Previous work exploring the effects of angiotensin II and its antagonists on anxiety-like behaviour has also indicated a time-dependent response, suggesting some element of 'rebound' effect at 3 h post-dose, and a complex interplay of AT_1 and AT_2-mediated effects [47]. Evidence of reduced anxiety-like behaviour 27-h after a predictably anxiogenic dose of angiotensin IV is therefore novel.

Many studies have looked at the effect of PAE in spatial response and memory in rodents. For example, a study in C57BL/6J mice has found long lasting learning and memory deficits in delay fear conditioning, trace fear conditioning and radial-maze tasks with no changes in spontaneous locomotor activity in 90 and 150 day old male offspring [24]. However, there have been very few studies looking at PAE and novel object recognition, with Popović et al. [51] showing that pre and postnatal exposure to 5% ethanol in Wistar rats caused a significant impairment in spatial learning and object recognition. Another study in C57BL/6J mice showed that prenatal exposure to 25% ethanol on gestational day 8 caused significant spatial memory impairments as well as impaired object recognition in 60 day old mice (52). The aim of the current study was to explore the effects of PAE on the pro-cognitive effects of angiotensin IV as determined by novel object recognition.

The mechanism of the pro-cognitive effect of angiotensin IV is unclear but it is known that it binds to insulin-regulated aminopeptidase (IRAP) [53]. IRAP possesses aminopeptidase activity and is responsible for the cleavage of endogenous peptides such as vasopressin and oxytocin but it is also associated with the GLUT4 glucose transported and, in combination with insulin, increases glucose uptake into cells; angiotensin IV inhibits the enzyme activity but enhances glucose uptake [reviewed in 54]. Harding and Wright, however, argue that another potential mechanism of action is dependent on activation of the hepatocyte growth factor/c-MET system [55]. PAE prevents the pro-cognitive effects of angiotensin IV in male offspring, suggesting some disruption of the binding sites and/or processes underlying its procognitive effects, or possibly some disruption of the synthesis or enhancement of the metabolism of angiotensin IV itself. These possibilities warrant further investigation although it is known that PAE can impact insulin signalling molecules in guinea-pigs [56] and in another condition associated with memory deficit, Alzheimer's disease, plasma concentrations of the putative receptor for angiotensin IV, IRAP, has been shown to be decreased, as have aminopeptidases A, B and N, which are involved in synthesis of angiotensin IV [57]. The possibility therefore exists that PAE may induce deficits in learning and memory at least partially by disruption of the synthesis/action of endogenous angiotensin IV.

An additional underlying theme of this study has been the sex-dependent nature of the results seen. Few studies have used female progeny in learning and memory tests and neither Popović et al. [51] nor Summers et al. [52] discriminated between male and female progeny. Despite this lack of behavioural evidence, other studies have shown that PAE has gender specific effects, with long-term potentiation being reduced in the male but not in female progeny [58]. Furthermore, the renin angiotensin system, and in particularly ACE activity is reported to have sex differences [59]. Interestingly a recent study, using a model of attention deficit disorder, has also shown an effect of ACE inhibitors in male but not female mice, giving further evidence for sex differences in the brain renin-angiotensin system [60]. In the current study, anxiety-like behaviour as determined by the elevated plus maze conducted at the conclusion of two-day behavioural test-battery for the animals, indicated reduced anxiety in male offspring only. In the open field test, conducted at the start of the test battery, the results suggested elevated anxiety induced by PAE in male offspring only. Recent evidence in humans has indicated that FASD is more prevalent in males than females [61], the results of this mouse model of PAE therefore appears to be reflective of the human condition in that some degree of sex-dependence has been identified.

In conclusion, the results of this study suggest that administration of 5% ethanol via drinking water throughout pregnancy and lactation in mice is a reflective model of FASD in humans and that the results suggests that PAE disrupts the actions of exogenous angiotensin IV which may be a mechanism responsible for the impaired learning and memory and possibly a future target for potential therapies.

Conflict of interests

The authors have no conflict of interests.

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SF performed the research, data analysis and prepared the manuscript; CS, AT, AM and TT performed the research; PRG designed the research study and edited the manuscript. AQ performed the blood

alcohol analyses.

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Highlights

- Prenatal exposure to 5% ethanol increased anxiety in male but not female progeny.
- Prenatal exposure to 5% ethanol abolished the precognitive effects of angiotensin IV in male progeny.
- Angiotensin IV induced anxiolysis in males exposed to 5% ethanol *in utero*.

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