Contents lists available at ScienceDirect

Neuropeptides

journal homepage: www.elsevier.com

Decreased behavioural and neurochemical effects of angiotensin IV following prenatal alcohol exposure in the mouse

Sara Fidalgo , Mira Patel , Angela Quadir , Wafia Sadiq , Paul R. Gard *

School of Pharmacy and Biomolecular Sciences, University of Brighton, Moulsecoomb, Brighton BN2 4GJ, UK

ARTICLE INFO

Keywords:
Prenatal alcohol
Angiotensin IV
Insulin-regulated aminopeptidase
Learning
Anxiety
Memory

ABSTRACT

Angiotensin IV (ang IV) is known to improve learning and memory in animal models but the mechanism is unclear. We have previously demonstrated sex differences in the pro-cognitive effects of ang IV, and that prenatal alcohol exposure (PAE) abolishes these effects. This study aimed to explore a possible mechanism underlying the sex differences and the effects of PAE in male mice.

Mouse breeding harems received 5% ethanol in drinking water throughout pregnancy and lactation in a two-bottle schedule. The effects of ang IV were assessed in offspring at 4 months of age using the open field test, novel object recognition test and elevated plus maze. Aminopeptidase activity of brain insulin-regulated aminopeptidase (IRAP), a putative target of ang IV, was determined.

As seen in a previous similar study, ang IV administered immediately after the second training trial significantly improved novel object recognition 24 h later in male mice but not female. PAE abolished this pro-cognitive effect in males. PAE also increased anxiety-like behaviour in male but not female offspring. Ang IV decreased the aminopeptidase activity of brain IRAP in control male, but not female, mice; PAE abolished this inhibitory effect.

Ang IV improved memory consolidation in male but not female mice and PAE abolished this effect in the males. While the effects of PAE may be related to increased anxiety; ang IV decreased the aminopeptidase activity in male but not female mice and PAE abolished this inhibitory effect. The results therefore suggest that improvements in learning and memory induced by peripheral administration of ang IV correlate with a reduction of the enzyme activity of IRAP. This is the first demonstration that ang IV administered peripherally can induce long-term (24 h) changes in IRAP function which are probably not simple competitive inhibition and the first demonstration that PAE alters IRAP activity.

1. Introduction

Angiotensin IV (ang IV) is an endogenous hexapeptide component of the renin-angiotensin system, in the brain it is produced in neurons and acts on specific receptors, AT_4, located in the cortex, hippocampus and basal ganglia (Jackson et al., 2018). When administered exogenously it has been shown to have beneficial effects on memory acquisition and recall in rats and mice (Braszko et al., 1988; Golding et al., 2010). Most workers administer the drug intracerebroventricularly (Wright et al., 1996; Paris et al., 2013), but we are the only group to use subcutaneous administration and to have demonstrated an effect of ang IV on memory consolidation, 24 h after administration (Golding et al., 2010).

The mechanism of the pro-cognitive effect of ang IV is unclear but it is known to bind to insulin-regulated aminopeptidase (IRAP) (Albiston et al., 2003), which has been proposed as the AT_4 receptor. 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 transporter. In combination with insulin, IRAP increases glucose uptake into cells. Ang IV inhibits the aminopeptidase activity of IRAP but enhances glucose uptake. One hypothesis suggests that the pro-cognitive effects of ang IV are a consequence of increased neuronal glucose uptake whilst an alternate hypothesis posits inhibition of aminopeptidase activity resulting in accumulation of endogenous substrates such as oxytocin and vasopressin which have both been shown to facilitate learning and memory (reviewed in Vanderheyden, 2009). Harding and Wright, meanwhile,

* Corresponding author.
Email addresses: A.Quadir@brighton.ac.uk (A. Quadir); P.R.Gard@brighton.ac.uk (P.R. Gard)

https://doi.org/10.1016/j.npep.2019.05.002
Received 26 February 2019; Received in revised form 26 April 2019; Accepted 1 May 2019
Available online xxx
0143-4179/

propose another potential mechanism of action: activation of the hepatocyte growth factor/c-MET system (Benoist et al., 2014) and Chow et al. (2015) have proposed that the action is via protein kinase Czeta. It is known that dopamine receptor antagonists and oxytocin receptor antagonists are able to block the pro-cognitive effects of ang IV (Braszko et al., 2008; Gard et al., 2007); oxytocin receptor antagonists also block the behaviour effects of LVV-hemorphin-7, another endogenous agonist of IRAP (Da Cruz et al., 2017).

As outlined above, our previous work has demonstrated that subcutaneous administration of ang IV immediately after the second learning trial of the novel object recognition task significantly improves novel object recognition 24 h later in male mice Golding et al., 2010). This procognitive effect of ang IV was not seen in age-matched female mice (Fidalgo et al., 2017). Furthermore prenatal exposure to low-dose ethanol abolished the effects of ang IV in adolescent male mice (ibid.). The aims of the current study were to explore sex differences in aminopeptidase activity of IRAP and the effects of prenatal ethanol exposure to ascertain whether differences may explain the observed differential effects of ang IV on behaviour, learning and memory.

2. Materials and methods

2.1. Animal husbandry

All procedures were approved by the University of Brighton Animal Welfare And Ethics Review Board, were licensed under EU directive 2010/63/EU and complied with the ARRIVE guidelines. C57BL/6 J mice were maintained at 19.0 ± 1 °C, 55% humidity and fed ad libitum on either a breeding diet (RM3 (E) 801,002 chow, Special Diet Services) (breeding harems) or a maintenance diet (RM1 (E)801,002 chow, Special Diet Services) (offspring). The mice were maintained on a 12-h light/ dark schedule, lights on 0700 h (60 Lux at cage level). Peri-natal mortality was monitored in all cages.

2.2. Pre-natal alcohol exposure (PAE)

Breeding harems were established with one male to 3–4 females; males were removed from the cages once pregnancy had been confirmed. Following an adaptation of the maternal ethanol consumption model described by Kleiber et al. (2011) the harems received fluid ad libitum under a two-bottle choice. The alcohol exposure groups had 24 h access to both a bottle of 5% ethanol sweetened with 0.066% $^{w}/_{v}$ saccharin solution and a bottle of tap-water. The control group had 24 h access to a bottle of 0.066% $^{w}/_{v}$ saccharin solution and a bottle of tap-water. In three control (saccharin) and four ethanol cages the volume of liquid consumed by each harem was recorded daily once pregnancy had been confirmed and the males had been removed from the cages. 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 approximately 4 months of age.

2.3. Drug administration

Ang IV (Val-Tyr-Ile-His-Pro-Phe-OH) was obtained from Bachem, (Germany). The stock solution of 1 mM (775 μg/mL) was stored frozen as 1 mL aliquots and was diluted in normal saline as required. The drug was administered subcutaneously (s.c.) in a volume of 10 mL/kg; the dose administered was 5.0 μg/kg. The vehicle control was normal saline.

2.4. Behavioural tests

Behavioural testing was conducted between 1000 and 1500 h. 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 ang IV or vehicle control.
Time 26 h: 3-min novel object discrimination period.
Time 27 h: 3-min elevated plus maze.

Details of the behavioural methods were identical to those reported in Fidalgo et al. (2017). In the open field test 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). For novel object recognition Ethovision software was used 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. Object recognition data were excluded for any animal that failed to spend any time exploring one of the objects, total locomotor activity data were retained.

For the elevated plus maze the software tracked total distance moved during each trial and the time spent in both open and closed arms. Open / closed arm data were excluded for any animal that failed to enter either one of the open or one of the closed arms.

2.5. Blood biochemistry

2.5.1. Maternal blood alcohol concentration determination

Blood was collected by cardiac puncture post mortem from a small sample of female ex-breeders maintained on the ethanol dosing schedule between 1100 h and 1300 h. Blood alcohol concentration was determined by gas chromatography as described in Fidalgo et al. (2017).

2.5.2. Blood glucose determinations

Blood glucose concentration was determined in a small sample of off-spring using a commercially-available glucose electrode (Accu-Chek). Briefly, following completion of the behavioural test battery, blood was collected by cardiac puncture immediately post-mortem between 1500 h and 1700 h and blood glucose concentration determined following manufacturer's instructions.

2.6. Brain biochemistry

2.6.1. Tissue preparation

Whole brains were removed immediately post-mortem and rapidly placed on ice before being stored at −80 °C. Stored brains were allowed to thaw before being placed into ice-cold extraction buffer (50 mM Tris, 140 mM NaCl and 250 mM Sucrose, pH 7.4) and homogenized at 20500 rpm for 10 s followed by 11,500 rpm for 15 s (IKA-T10 basic ultra-turrax). The homogenized samples were centrifuged at 4000*g* for 8 min to remove any cellular debris, and the supernatant further centrifuged at 100,000*g* for 40 min in order to pellet the membrane fragments. The pellet was washed by resuspension in 1 ml of extraction buffer and a 10 μL sample removed prior to a final centrifugation at 100,000*g* for 40 min. The protein content of the 10 μL sample membrane-fragment suspension and the supernatant was determined using the Bradford method (Bradford, 1976). The final membrane pellet was resuspended in assay buffer (50 mM Tris and 140 mM NaCl, pH 7.4) to give a protein concentration of 1 μg/μL.

2.6.2. Determination of brain insulin-regulated aminopeptidase (IRAP) activity

Aminopeptidase activity of IRAP was assessed by the cleavage of L-leucine-p-nitroanilide (LPN) to L-leucine and p-nitroaniline (Stragier et al., 2007). Briefly, 50 µg of membrane pellet preparation was incubated with 0–3.0 mM LPN in a total volume of 250 µL at 37 °C. The formation of the yellow-coloured p-nitroaniline was monitored using a Biotek plate reader and Gen 5 software at 10 min intervals over 60 min. The average initial rate of product formation (mMol/min) was determined for each of the concentrations of LPN by reference to a p-nitroaniline standard curve and Vmax and Km values determined by Michaelis-Menten analysis (using GraphPad Prism5 software).

2.7. Data analysis

All data sets were tested for normality using the Kolmogorov-Smirnov test. Only data for fluid intake and blood glucose were consistently normally distributed, hence non-parametric procedures were used as appropriate. Data for the fluid intake are presented as means ± standard error of the mean (sem), data for the blood glucose are presented as a scatter graph with mean values indentified. Group means for parametric data were compared using Student's independent *t*-test or analysis of variance (ANOVA) as appropriate.

For non-parametric data derived from the behavioural assessments the results are expressed as scatter plots with median values indicated. Group means were compared using Mann-Whitney, Wilcoxon or Kruskall-Wallis tests as appropriate. Where 2- or 3-way ANOVA was most appropriate, the non-parametric data were transformed by ranking and the tests undertaken on the ranked data (Oliver-Rodriguez and Wang, 2015). All statistical tests were performed using GraphPad Prism with the exception of the 2- and 3-Way ANOVA on transformed data which used Mintab 17.

Data for aminopeptidase activity was analysed using 2-way repeated measures ANOVA and fitted to the Michaelis-Menten equation using GraphPad Prism. Data were also analysed using the F-test which determines whether fitting two Michaelis-Menten curves to the data sets is superior to fitting of a single curve. In the case that two curves are superior, an independent *t*-test was used to determine whether either the Vmax or Km estimates were significantly different or both.

Survival data of the different litters was compared using the Chi-squared test (Minitab 17).

Sample size calculations were based on behavioural data from the novel object recognition test. A minimum group size of 8 gives a test power of 80% with α at 0.05. A p value $<.05$ was considered to be statistically significant.

3. Results

3.1. Pre-natal alcohol exposure (PAE)

Fig. 1 illustrates that for the saccharin control group over 10 weeks the average water intake was 0.85 ± 0.15 mL per mouse per day and 8.86 ± 0.61 mL saccharin solution per mouse per day. For the prenatal alcohol group, the respective figures were 1.26 ± 0.22 mL water and 4.92 ± 0.26 ethanol/saccharin solution per mouse per day. In total, therefore, the saccharin control group consumed a significantly greater daily volume of fluid than the PAE group (9.70 ± 0.86 versus 6.11 ± 0.26 mL; $p = .0008$, Student's t-test).

Five breeding cages of animals (two saccharin control and three ethanol in saccharin) were monitored for perinatal deaths. Of the 95 animals born to dams receiving saccharin alone, 70 (73%) survived to weaning and of the 189 animals born to dams receiving ethanol plus saccharin, 125 (66%) survived to weaning. This difference was non-significant (Chi-squared test).

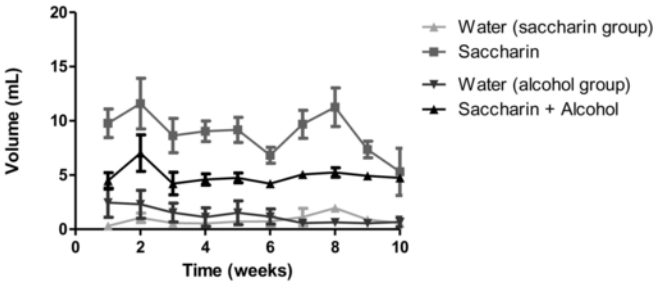

Fig. 1. Average daily intake of water, 0.066% $^{w}/_{v}$ saccharin solution or 5% ethanol in 0.066% $^{w}/_{v}$ saccharin solution over 10 weeks by control ($n = 3$) and prenatal alcohol exposure breeding harems ($n = 4$); volumes are expressed mL per adult mouse.

Behavioural and biochemical testing of offspring occurred at mean age 124 ± 6 days. Within any particular experiment ages were closely matched. The age-matched weights of the male off-spring were 27.47 ± 0.97 g ($n = 37$) for the control group and 28.53 ± 0.55 g ($n = 38$) for the PAE group. Respective values for age-matched females were 24.41 ± 0.42 g ($n = 28$) and 24.25 ± 0.66 g ($n = 21$). PAE therefore had no significant effect on body weight in either male or female offspring.

3.2. Behavioural tests

3.2.1. Open field test

In the open field test, male control mice showed significantly greater locomotor activity than female mice ($p = .0006$), although there were no significant sex differences in thigmotaxis.

PAE resulted in significantly decreased locomotor activity in the male offspring ($p < .005$), but there was no significant effect in female offspring. Similarly, with respect to thigmotaxis, PAE resulted in significantly greater thigmotaxis in the male offspring ($p < .0001$), with no significant effect in female offspring (Fig. 2).

3.2.2. Novel object recognition

In the novel object recognition test in male offspring, control animals (offspring of dams receiving a choice of saccharin and water) did not differentiate between the familiar and novel objects following treatment with saline after the second learning trial. Control animals treated with ang IV after the second learning trial, however, significantly differentiated between the familiar and novel objects 24 h later ($p < .005$, Wilcoxon test, Fig. 3), spending approximately twice as long exploring the novel rather than the familiar object. In male offspring that had been exposed to prenatal ethanol, again there was no significant differentiation between familiar and novel objects in those animals treated with saline vehicle after the second learning trial, but now there was no significant differentiation by those animals treated with ang IV after the second learning trial (Fig. 4).

Analysis of total locomotor activity during the recall phase of the novel object recognition test investigated the effects of PAE, the effects of ang IV delivered after the second learning trial (24 h earlier) and any interaction. The results indicated that treatment with ang IV had no significant effect on locomotor activity, but that PAE induced a significant increase in locomotor activity ($p < .018$, ANOVA). There was no significant interaction.

In the female offspring, ang IV had no significant effect on novel object recognition in either control or PAE animals and there was no significant effect of ang IV or PAE on locomotor activity.

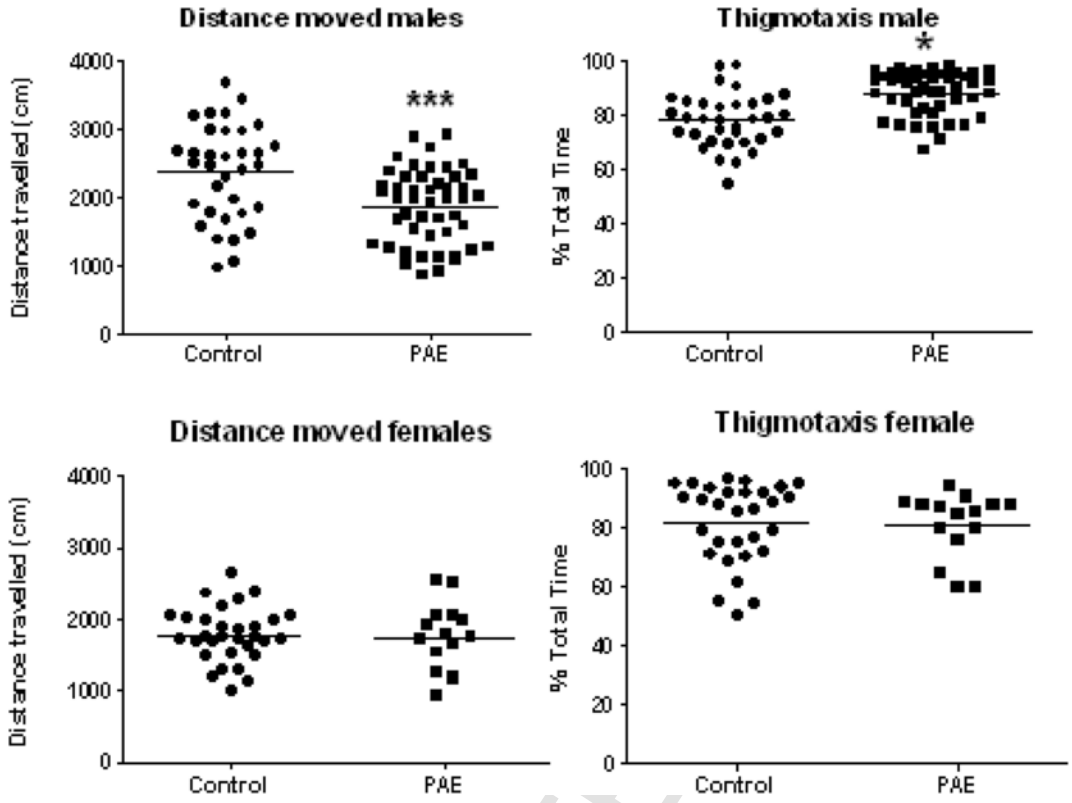

Fig. 2. The effects of PAE on open field locomotion and thigmotaxis in male and female off-spring. * and *** represent $p < .05$ and 0.005 respectively (Mann-Whitney), n = at least 15 in all groups.

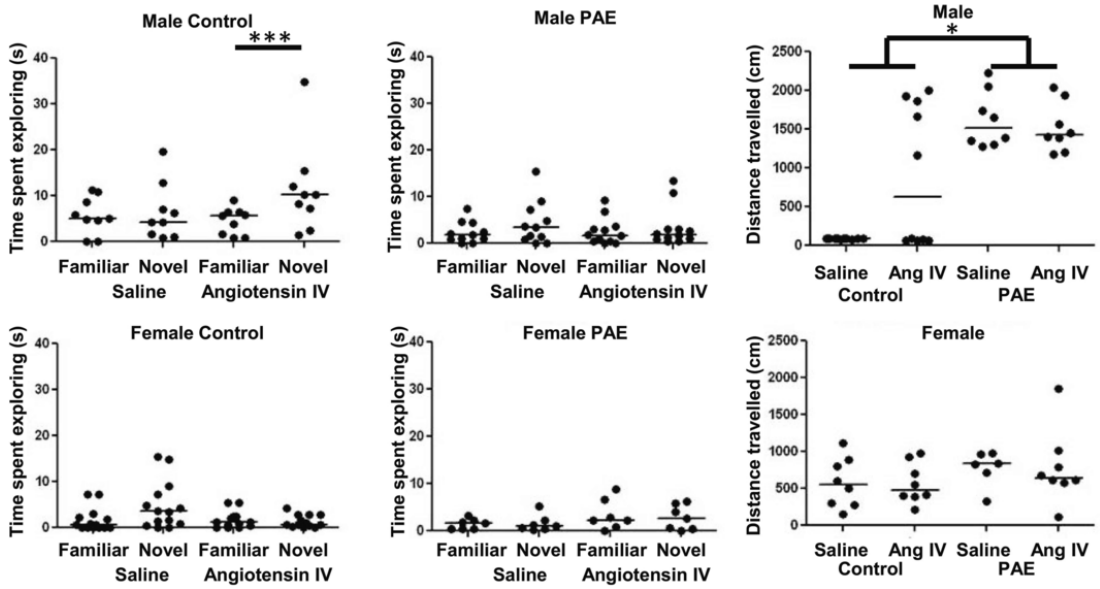

Fig. 3. Differentiation between novel and familiar objects and total distance moved in the novel object recognition test by male and female offspring exposed prenatally to either saccharin (control) or ethanol and saccharin (PAE) and then subsequently treated with ang IV (5 μg/kg, s.c.) or vehicle control after the second learning trial. * & **** represents statistically significant differences between groups, $p < .05$ and 0.0005 respectively (Wilcoxon and ANOVA tests as appropriate). Group sizes 6–14.

3.2.3. Elevated plus maze

In the elevated plus maze neither PAE, nor subsequent treatment with ang IV (as part of the novel object recognition test) had any significant effect on total distance moved nor proportion of time spent on the open arms in either male or female offspring. There were no signifi-

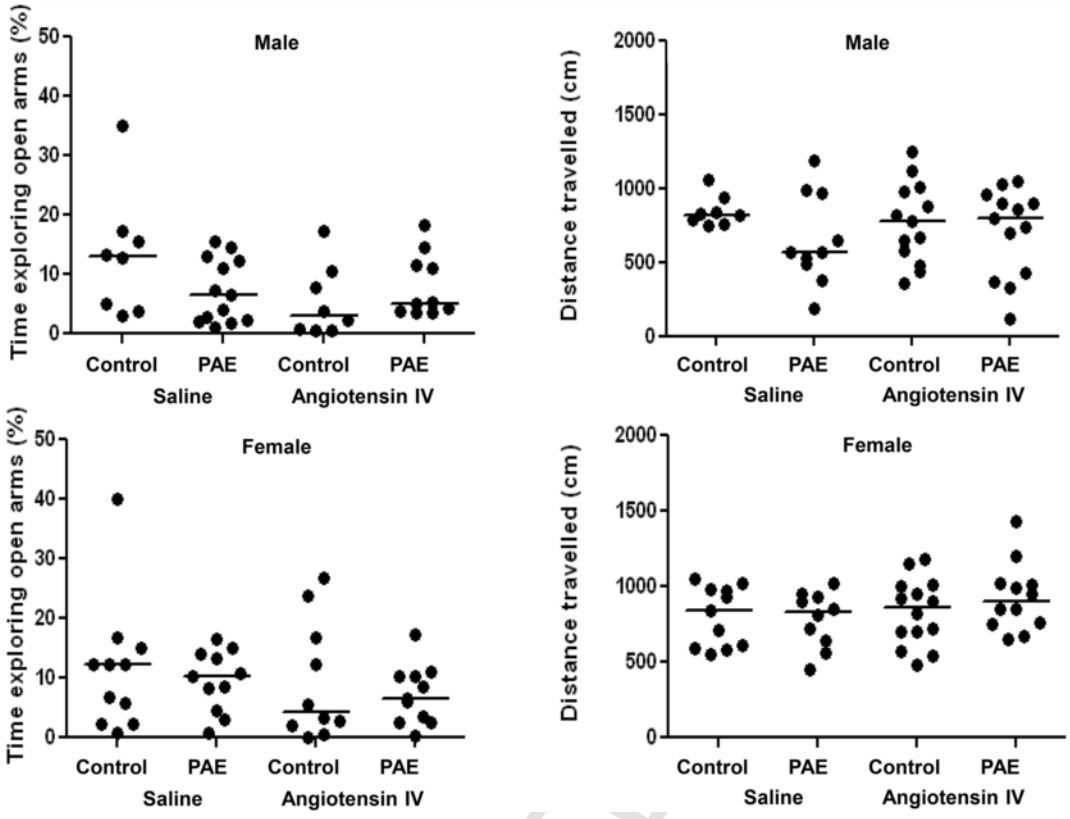

Fig. 4. Proportion of time spent on the open arm of, and total distance travelled on, the elevated plus maze by male and female offspring exposed prenatally to either saccharin (control) or ethanol and saccharin (PAE) and then subsequently treated with ang IV (5 μg/kg, s.c.) as part of the novel object recognition test. Group sizes 8–13.

cant differences between males and females for total distance moved nor time spent on the open arms.

3.3. Blood biochemistry

3.3.1. Maternal blood alcohol concentration determination

Only 3 of the 5 maternal blood samples tested had measurable blood alcohol concentrations. The three values were 1.885, 6.549 and 4.793 mg/dL. The mean of the five female breeding females sampled was therefore 2.645 ± 1.312 mg/dL.

3.3.2. Blood glucose determinations

Male offspring had significantly higher blood glucose concentrations that female mice ($p = .006$) but PAE had no significant effect on the blood glucose concentration in either male or female mice, nor was there any sex X pre-treatment interaction (2-way ANOVA). (Fig. 5).

Fig. 5. Blood glucose concentrations in male and female offspring exposed pre-natally to either saccharin (control) or ethanol and saccharin (PAE) (n = 4–8).

3.4. Determination of brain IRAP activity

Data for rates of product formation at different substrate concentrations were fitted to the Michaelis-Menten equation and the F-test used to determine whether fitting two Michaelis-Menten curves to the data sets of the different treatment groups was superior to fitting of a single curve.

Initial comparisons considered only those animals treated with saline as part of the novel object recognition test and explored the effects of PAE. In niether male nor female mice was there a significant effect of PAE on the Michaelis-Menten parameters as determined by 2-way ANOVA, and in both cases the F-test determined that the combined data were better described by a single Michaelis-Menten curve than by two separate curves. These results indicate that PAE alone has no effect on enzyme characteristics.

The effects of ang IV administration as part of the novel object recognition test was then explored. In the case of the male saccharin group, the 2-way ANOVA indicated a significant main effect of substrate concentration ($p < .0001$), no significant effect of ang IV but a significant drug treatment x substrate concentration interaction ($p = .0196$). The F test indicated that fitting two Michaelis-Menten curves was superior to a single curve ($p < .0001$). These results indicate that ang IV significantly alters enzyme characteristics. *t*-tests indicated that the was no significant difference in the estimated Km, but that the estimates of Vmax were significantly different ($p = .0182$).

In male PAE animals, 2 way-ANOVA indicated that the Michaelis-Menten data were not significantly affected by administration of ang IV. Similarly in female offspring, 2-way ANOVA indicated that neither PAE nor ang IV had any effect on enzyme activity and the F-test indicated that the data for these groups were best fitted to a single Michaelis-Menten curve, i.e. in PAE animals, ang IV had no effect on

enzyme characteristics; the were no significant differences in Km nor Vmax (See Fig. 6).

4. Discussion

Our findings indicate that ang IV (5 μg/kg, s.c.) administered immediately after the second training trial significantly improved novel object discrimination 24 h later in male control mice but not female mice. Ang IV thus appears to enhance memory consolidation in male mice but not female mice. Before considering any neurochemical basis of the sex difference, the possible behavioural confounding features will be explored. At the outset of the study the open field test showed that female control mice had lesser locomotor activity than the male mice, but there was no significant difference in thigmotaxis. Reduced locomotor activity and increased thigmotaxis are typically seen as anxiety-like behaviour, such results therefore suggest the possibility of some increased anxiety in the female mice, although not sufficient to influence thigmotaxis.

The elevated plus-maze is generally considered to be superior to locomotor activity and thigmotaxis in the assessment of anxiety-like behaviour. This test was conducted on the second day of the test battery, immediately after the novel object recognition test and is therefore probably more reflective of anxiety at the time that recall was being assessed. Decreased distance moved and decreased time spent on the open arms are indicative of anxiety-like behaviour. The results show that males and females did not differ in their anxiety-like behaviour and that pre-treatment with ang IV had no effect on anxiety-like behaviour. The sex differences in the effect of ang IV on memory consolidation can therefore not be explained by a confounding influence of anxiety at the time of recall.

Turning to a biochemical explanation of the observed sex differences in the effects of ang IV on memory consolidation, it important to note that female C57BL/6 mice have previously been reported to exhibit estrous cycle specific behaviour in the novel object recognition task and to be refractory to the analgesic effect of ang IV. Cordeira et al. (2018) reported that female mice exhibited preferential exploration of the novel object in the novel object recognition task only during the proestrus and estrous phase of the estrous cycle, not during metestrous or diestrous. Chow et al. (2018) studied the analgesic effects of intrathecal ang IV, oxytocin and LVV-hemorphin-7 in male and female C57Bl/6 mice and found that the drugs were effective in males but 'either extremely weak or absent in female mice at the same dose'. We did not assess estrous cycle stage in the current study, but the work of Chow and colleagues suggests that females differ from males in the activity of IRAP in that ang IV, oxytocin and LVV-hemorphin-7 are all ligands for IRAP. Assessment of aminopeptidase activity of IRAP in brain tissue harvested from control animals of the current study one hour after the recognition trial revealed that male and female mice did not differ significantly. Twenty-four hour pre-treatment with ang IV, however, significantly reduced the aminopeptidase activity in male mice but not in female mice. It is thus apparent that effects of ang IV on IRAP aminopeptidase function are sex-dependent and that the improved memory consolidation is correlated with the reduced enzyme function.

The relationship between enzyme activity and memory consolidation is further highlighted by the effects of the prenatal alcohol exposure (PAE). The dose of ethanol used in this study is at the lower end of the spectrum for such studies (see Petrelli et al., 2018), and there were no effects of this dose on litter survival rate or off-spring body weight; 32% litter loss is typical for this strain of mouse (Weber et al., 2013). We chose to study the effects of PAE in 124-day old animals as we are

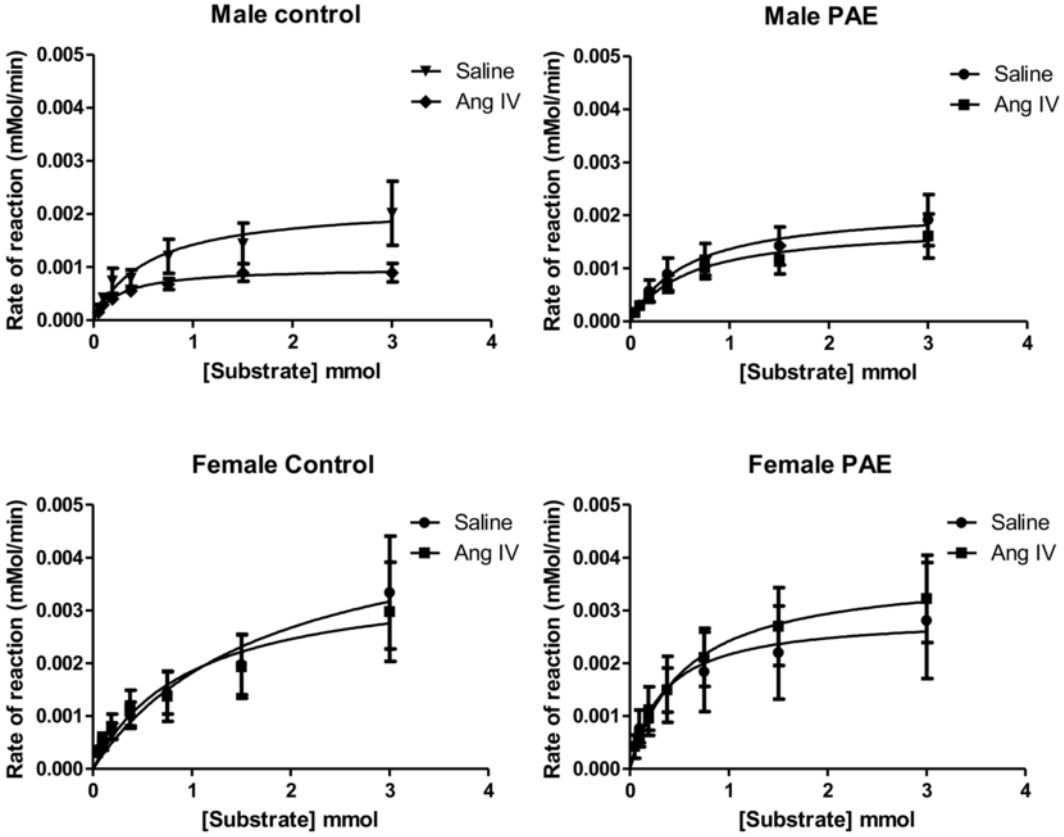

Fig. 6. Michaelis-Menten curves for membrane-bound insulin-regulated aminopeptidase activity in brain tissue from male and female mice exposed pre-natally to either saccharin (control) or ethanol and saccharin (PAE) and subsequently treated with either saline or ang IV. There were no significant effects of pre-natal alcohol exposure on enzyme activity in those animals subsequently treated with saline. Angiotensin IV treatment caused a significant decrease in Vmax but not Km in saccharin exposed males only ($p = .0182$). Group size = 6–8.

ultimately interested in the long-term effects in children with fetal alcohol spectrum disorder (FASD) and 124 days is within normal range used in mouse models of PAE. Those male mice exposed to prenatal ethanol failed to show the improved memory consolidation following ang IV administration at approximately 4 months of age, and at the same time, the pre-treatment with ang IV failed to reduce the aminopeptidase activity of IRAP in PAE male mice. Taken overall, the results of the male and female control and PAE mice therefore demonstrate that improved memory consolidation only occurs in those animals where ang IV is seen to decrease aminopeptidase function of IRAP 24 h after administration suggesting a possible causative relationship.

The likelihood of peripherally administered ang IV surviving plasma peptidases, crossing the blood-brain barrier and inhibiting cortical or hippocampal IRAP has been questioned previously (Ho and Nation, 2018). The results presented here, however, indicate some effect. The results highlight the fact that ang IV, administered subcutaneously, was able to significantly decrease the Vmax of IRAP in male mice, suggesting that the peptide was able to cross the blood brain barrier and reach sufficient concentrations to have an effect. It is improbable that this result reflects competitive inhibition of the enzyme due both to the concentration of ang IV that would be needed (Demaegdt et al., 2012), but also the expectation that bound ang IV would dissociate from the enzyme during membrane fragment preparation. The most likely mechanism would be that ang IV induced IRAP internalisation following binding, as shown by Demaegdt et al. (2011) with the internalised vesicles not being isolated by the membrane fragmentation/centrifugation process. This IRAP internalisation process would be associated with enhanced glucose uptake by the cells together with decreased membrane-bound aminopeptidase activity. It is known that glucose administration has the ability to improve learning and memory (Messier, 1997) and while the current study did not explore the effects of PAE on neuronal glucose, the results indicate definitively that there was no lasting effect on blood glucose homeostasis. Whether improved memory consolidation is associated with inhibition of aminopeptidase activity or enhanced glucose uptake is therefore still unresolved.

Importantly, enhancement of memory consolidation by ang IV was only seen in those animals in which there was also decreased Vmax of IRAP by ang IV, suggesting a causative relationship. In female offspring there was neither enhancement of cognition, nor inhibition of aminopeptidase activity.

Of course, the potential confounding effects of anxiety on memory consolidation and recall warrant consideration. Those male mice exposed to ethanol in utero exhibited significantly less locomotor activity in the open field and greater thigmotaxis, than their unexposed counterparts. These results are indicative of greater anxiety at the start of the behavioural test battery. Such increased anxiety could explain the impaired learning and memory. Results of the elevated plus maze at the end of the battery, however, showed no significant effects of PAE (nor ang IV pre-treatment) on anxiety as assessed by total distance moved and time spent on the open arms of the maze. It is therefore unlikely that increased anxiety associated with PAE interfered with memory recall, but earlier elevated anxiety may have interfered with memory acquisition or consolidation.

One more potential explanation of the observed results remains: purely pharmacokinetic. In male mice PAE may induce peptidase metabolism of ang IV, thus abolishing the effects of the ang IV dose given peripherally. Similarly in the female mice there may by a sex difference in peptidase degradation of ang IV rendering the dose given inactive in control mice, with no addition apparent effect of PAE. An investigation of this proposal would be possible by determination of plasma and brain ang IV pharmacokinetics in male and female control and PAE mice, although the findings of Chow et al., 2018 indicate that there are sex differences in responses to intrathecal ang IV suggesting that such sex differences are not dependent on pharmacokinetic differences.

In conclusion, our study found that there was a sex difference in the effects of peripherally administered ang IV on memory consolidation, and that prenatal exposure to low dose ethanol abolished the precognitive effects of ang IV. Whilst the sex difference and effect of PAE might be explained by induced anxiety or increased peptide metabolism, the most likely explanation is probably a difference in the aminopeptidase activity of IRAP with improved memory consolidation correlated with decreased enzyme Vmax probably as a consequence of enzyme internalisation. This study is the first to demonstrate an effect of peripherally administered ang IV of IRAP function.

Acknowledgements

This work was supported by the European INTERREG IVA (project number 4230), named Peptide Research Network of Excellence (PeReNE). The authors would also like to acknowledge the help of the staff at the bioresources unit at the University of Brighton.

SF performed / supervised the research and data analysis; MP and WS performed the IRAP studies and AQ undertook the blood ethanol analyses. PRG designed the research study, analysed the data and wrote the manuscript.

Conflict of interests

The authors have no conflict of interests.

References

Albiston, A.L., Mustafa, T., McDowall, S.G., Mendelsohn, F.A.O., Lee, J., Chai, S.Y., 2003. AT(4) receptor is insulin-regulated membrane aminopeptidase: potential mechanisms of memory enhancement. Trends Endocrin. Met. 14, 72–77.

Benoist, C.C., Kawas, L.H., Zhu, M., Tyson, K.A., Stillmaker, L., Appleyard, S.M., Wright, J.W., Wayman, G.A., Harding, J.W., 2014. The procognitive and synaptogenic effects of angiotensin IV-derived peptides are dependent on activation of the hepatocyte growth factor/c-MET system. J. Pharmacol. Exp. Ther. 351, 390–402.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Braszko, J.J., Kupryszewski, G., Witczuk, B., Wiśniewski, K., 1988. Angiotensin II-(3–8)-hexapeptide affects motor activity, performance of passive avoidance and a conditioned avoidance response in rats. Neurosci. 27, 777–778.

Braszko, J.J., Wielgat, P., Walesiuk, A., 2008. Effect of D(3) dopamine receptors blockade on the cognitive effects of angiotensin IV in rats. Neuropeptides 42, 301–309.

Chow, L.-H., Tao, P.-L., Chen, Y.-H., Lin, Y.-H., Huang, E.Y.-K., 2015. Angiotensin IV possibly acts through PKMzeta in the hippocampus to regulate cognitive memory in rats. Neuropeptides 53, 1–10.

Chow, L.-H., Chen, Y.-H., Lai, C.F., Lin, T.Y., Chen, Y.J., Kao, J.H., Huang, E.Y.-K., 2018. Sex difference of angiotensin IV-, LVV-hemophin 7-, and oxytocin-induced antiallodynia at the spinal level in mice with neuropathic pain. Anesth. Analg. 126, 2093–2101.

Cordeira, J., Kolluru, S.S., Rosenblatt, H., Kry, J., Strecker, R.E., McCarley, R.W., 2018. Learning and memory are impaired in the object recognition task during metestrus/diestrus and after sleep deprivation. Behav. Brain Res. 339, 124–129.

Da Cruz, K.R., Turones, L.C., Camargo-Silva, G., Gomes, K.P., Mendonça, M.M., Galdino, P., Rodrigues-Silva, C., Santos, R.A.S., Costa, E.A., Ghedini, P.C., Ianzer, D., Xavier, C.H., 2017. The hemoglobin derived peptide LVV-hemorphin-7 evokes behavioral effects mediated by oxytocin receptors. Neuropeptides 66, 59–68.

Demaegdt, H., Gard, P.R., De Backer, J.P., Lukaszuk, A., Szemenyei, E., Toth, G., Tourwe, D., Vauquelin, G., 2011. Binding of "AT4 receptor" ligands to insulin regulated aminopeptidase (IRAP) in intact Chinese hamster ovary cells. Mol. Cell. Endocrinol. 339, 34–44.

Demaegdt, H., De Backer, J.P., Lukaszuk, A., Toth, G., Szemenyei, E., Tourwe, D., Vauquelin, G., 2012. Angiotensin IV displays only low affinity for native insulin-regulated aminopeptidase (IRAP). Fundam. Clin. Pharmacol. 26, 194–197.

Fidalgo, S., Skipper, C., Takyi, A., McIver, A., Tsiligkaridis, T., Quadir, A., Gard, P.R., 2017. Low-dose chronic prenatal alcohol exposure abolishes the pro-cognitive effects of angiotensin IV. Behav. Brain Res. 329, 140–147.

Gard, P.R., Daw, P., Sayyad Mashhour, Z., Tran, P., 2007. Interactions of angiotensin IV and oxytocin on behaviour in mice. J. Renin-Angiotensin Aldosterone System 8, 133–138.

Golding, B.J., Overall, A.D., Brown, G., Gard, P.R., 2010. Strain differences in the effects of angiotensin IV on mouse cognition. Eur. J. Pharmacol. 641, 154–159.

Ho, J.K., Nation, D.A., 2018. Cognitive benefits of angiotensin IV and angiotensin (1-7): a systematic review of experimental studies. Neurosci. Biobehav. Rev. 92, 209–225.

Jackson, L., Eldahshan, W., Fagan, S.C., Ergul, A., 2018. Within the brain: the renin angiotensin system. Int. J. Mol. Sci. 19, https://doi.org/10.3390/ijms19030876, article 876.

Kleiber, M.L., Wright, E., Singh, S.M., 2011. Maternal voluntary drinking in C57BL/6J mice: advancing a model for fetal alcohol spectrum disorders. Behav. Brain Res. 223, 376–387.

Messier, C., 1997. Object recognition in mice: improvement of memory by glucose. Neurobiol. Learn. Mem. 67, 172–175.

Oliver-Rodriguez, J.C., Wang, X.T., 2015. Non-parametric three-way mixed ANOVA with aligned rank tests. Brit. J. Math. Stat. Psy. 68, 23–42.

Paris, J.J., Eans, S.O., Mizrachi, E., Reilley, K.J., Ganno, M.L., McLaughlin, J.P., 2013. Central administration of angiotensin IV rapidly enhances novel object recognition among mice. Neuropharmacol. 70, 247–253.

Petrelli, B., Weinberg, J., Hicks, G., 2018. Effects of prenatal alcohol exposure (PAE): insights into FASD using PAE mouse models. Biochem. Cell Biol. 96, 131–147.

Stragier, B., Demaegdt, H., De Bundel, D., Smolders, I., Sarre, S., Vauquelin, G., Ebinger, G., Michotte, Y., Venderheyden, P., 2007. Involvement of insulin-regulated aminopeptidase and/or aminopeptidase N in the angiotensin IV-induced effect on dopamine release in the striatum of the rat. Brain Res. 1131 (77–105), .

Vanderheyden, P.M.L., 2009. From angiotensin IV binding site to AT(4) receptor. Mol. Cell. Endocrinol. 302, 159–166.

Weber, E.M., Algers, B., Würbel, H., Hultgren, J., Olsson, I.A., 2013. Influence of strain and parity on the risk of litter loss in laboratory mice. Reprod. Domest. Anim. 48, 292–296.

Wright, J.W., Clemens, J.A., Panetta, J.A., Smalstig, E.B., Weatherly, L.A.S., Kramar, E.A., Pederson, E.S., Mungall, B.H., Harding, J.W., 1996. Effects of LY231617 and angiotensin IV on ischemia-induced deficits in circular water maze and passive avoidance performance in rats. Brain Res. 717, 1–11.