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Prenatal ethanol exposure: Sex differences in anxiety and anxiolytic response to a 5-HT_{1A} agonist

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Abstract

This study utilized a novelty-induced suppression of feeding task to examine anxiety-like behaviour and the anxiolytic effects of the 5-HT_{1A} agonist, 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT), in rats prenatally exposed to ethanol. Adult offspring from ethanol exposed (E), pair-fed control (PF) and ad libitum-fed control (C) dams were habituated to a novel palatable food for 21 days and measures of baseline feeding obtained. On day 22 (d 22), animals received either 8-OH-DPAT (0.06 mg/kg) or vehicle (0.9% NaCl) and feeding behaviour in the home cage or a novel cage was observed. Factor analyses revealed that feeding behaviour on d 21 (habituation) and d 22 (test day) are reflective of two different affective states, and that the single factor that emerged for novel cage testing on d 22 likely reflects the anxiety evoked by the novel test condition. Analyses of variance on the variables loading significantly onto the factors support the suggestion that the novel environment is anxiogenic for both females and males, and that 8-OH-DPAT acts as an anxiolytic. However, although both females and males showed alterations in behaviours (latency, amount, duration of feeding) reflective of anxiety, 8-OH-DPAT had anxiolytic effects primarily in females. Importantly, prenatal ethanol exposure altered several aspects of behavior in this task. Both E females and males consumed less than their control counterparts on d 21, suggesting a possible delay or deficit in response habituation. During home cage testing on d 22, overall feeding rate was slower in E than in C females, and E males consumed less than PF and C males. In addition, a smaller percentage of E than PF and C females fed in the novel environment, and latency to feed was significantly increased in E compared to control females. These findings indicate that prenatal ethanol exposure results in increased anxiety-like behaviour in adulthood, and that prenatal ethanol-induced hyponeophagia may be, at least in part, mediated by the 5-HT_{1A} receptor. This study is one of the first to demonstrate specific increases in anxiety-like behaviour in animals prenatally exposed to ethanol, and further supports the utility of the novelty-induced suppression of feeding task in assessing anxiety and the effectiveness of anxiolytic agents.

Keywords: Prenatal ethanol exposure; Anxiety-like behaviour; 8-OH-DPAT; Serotonin; Novelty-induced suppression of feeding

1. Introduction

In-utero exposure to alcohol can disrupt development of many systems. In humans, heavy, chronic alcohol consumption during pregnancy can result in Fetal Alcohol Spectrum Disorder (FASD). Individuals with FASD can present with characteristic facial features and other physical abnormalities, growth deficiencies, cognitive impairments and behavioural problems, including hyperactivity, attentional deficits and a decreased ability to inhibit responding. Of particular relevance to our study, individuals with FAS and those with milder alcohol related effects typically exhibit a number of secondary disabilities including an increased incidence of psychiatric disorders, such as anxiety and depression. Compared to nonexposed children, Roebuck et al. (1999) found that alcohol-exposed children scored higher on the anxiety subscale of the Personality Inventory for Children as well as the anxious/depressed subscale of the Achenbach Child Behavior Checklist (Sood et al., 2001). In adults, Famy et al. (1998) found that 25 percent of the participants in their study with FAS or Fetal Alcohol Effects had at least one type of anxiety disorder.

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Rodents exposed prenatally to ethanol (E animals) demonstrate many of the same short and long-term deleterious effects observed in children prenatally exposed to alcohol and can therefore provide a useful experimental model. Initial studies discovered that E animals displayed growth deficiencies, physical abnormalities, and behavioural abnormalities similar to those observed in children with FASD, including hyperactivity and deficits in response inhibition (Riley et al., 1979a,b). E animals also show altered responses in several behavioural tests that suggest that the increased incidence of anxiety disorders following prenatal alcohol exposure in humans may also be modelled by prenatal ethanol exposure in rodents.

Two of the most popular tests of anxiety are the open field and the elevated plus-maze. In the open field, anxiety is typically reflected in decreased ambulation and/or decreased entries into the central area of the field. However, data have shown that prenatal ethanol exposure generally results in increased activity in the open field, as measured by increased ambulation and/or rears (Fernandez et al., 1983; Osborne et al., 1980; Bond and Di Giusto, 1976; Branchey and Friedhoff, 1976). Investigators have typically concluded that increased locomotor activity in E animals parallels the hyperactivity observed in children with FASD. Indeed, hyperactivity is one of the most common behavioural effects observed in E animals (Riley, 1990). However, one confound that may affect interpretation of results from the open field test of anxiety is that indices of activity level and emotionality often cannot be separated from those of exploratory behaviour (Weiss et al., 1998). This confound may contribute to the contradictory results that have been found in the open field, as others have failed to find an effect of prenatal ethanol exposure on activity in the open field (Nelson et al., 1988; Mothes et al., 1996; Osborn et al., 1998a,b), or have observed effects only in females (Grant et al., 1983). Previous studies have addressed these issues to some extent by utilizing tasks such as the hole board, where head-dipping and nose-poking provide more specific measures of exploration, separate from locomotor behaviour (e.g., Riley et al., 1979a,b). However, the utility of the open field in measuring anxiety-like behaviours has not been addressed by these alternative tasks.

Our laboratory was one of the first to conduct experiments employing the elevated plus-maze to study the effects of prenatal ethanol exposure on anxiety-like behaviour (Osborn et al., 1998a,b). Results from this widely used and validated test of anxiety are also somewhat inconclusive. Anxiety-like behaviour in ethanol exposed offspring appears to depend on sex, the variable examined, and whether the animal is naive to the test or has had prior experience with the open field (Osborn et al., 1998a,b). For example, the behaviour of female rats in this test appears to reflect activity or exploration, rather than anxiety, while the reverse is true for males (Fernandes et al., 1999). Data from our lab (Osborn et al., 1998a) demonstrate that, compared with PF and C animals, E males and females both exhibit higher levels of exploratory behaviours when placed directly on the plus maze from their home cages without prior exposure to an open field. After open field exposure, however, both E males and females had lower levels of exploratory behaviours than C males and females. Furthermore, E females showed an increase in fear-related behaviours on the plus maze compared with controls, regardless of prior open field exposure. Interestingly, E females and males both showed a greater anxiolytic response to diazepam in the plus-maze compared to their PF and C counterparts following prior open field exposure (Osborn et al., 1998b), suggesting that prenatal ethanol exposure alters the neurotransmitter receptors, specifically GABA receptors, targeted by anxiolytics.

The main objective of the present study was to examine the effect of prenatal ethanol exposure on anxiety-like behaviour in a novel anxiety task. We measured anxiety-like behaviour in adult female and male offspring from ethanol exposed (E), pair-fed control (PF) and ad libitum-fed control (C) dams in the novelty-induced suppression of feeding task (Merali et al., 2003; Muller-Gass et al., 2000), a test of anxiety that does not utilize exploration as an index of anxiety, and thus addresses the conflicting results on the effects of prenatal ethanol exposure on responses in the elevated plus-maze, open field and other behavioural tests. This test is based on an animal's natural reluctance to approach and consume a familiar, palatable food in a novel environment. This effect is termed hyponeophagia and is thought to reflect the anxiety induced by placing an animal in a conflict situation (i.e., fear of novel setting vs. drive to consume palatable food) (Poschel, 1971). Animals were habituated in their home cages to a novel food, graham crumbs (No Name™) for a period of 21 days, and habituation was assessed. Subsequently, animals were exposed to a novel bedding-free cage or retested in their home cage, and feeding behaviour was observed following 8-OH-DPAT or saline treatment. This test has recently been developed and validated for use with mice by Merali et al. (2003). These authors report that this test is sensitive to acute anxiolytic agents such as diazepam, chlordiazepoxide and propanolol, but not the non-anxiolytics, haloperidol and amphetamine. Other reports also support use of this modified paradigm as a test of anxiolytics as well as antidepressants (for review see Dulawa and Hen, 2005).

Our laboratory has previously observed increased 5-HT_{1A} receptor-mediated physiological function in E animals. That is, adult E females and males show increased hypothermia in response to the 5-HT_{1A} agonist 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-OH-DPAT) (Hofmann et al., 2002). The 5-HT_{1A} receptor has recently been found to play an especially important role in the expression of anxiety-like behaviour (Gross et al., 2002). For example, 5-HT_{1A} knockout mice exhibit increased anxiety in several tasks, including the novelty-induced suppression of feeding task (Gross et al., 2000). In addition, clinically and in the laboratory, 5-HT_{1A} agonists have anxiolytic effects. Therefore, a second purpose of the present study was to examine the anxiolytic effects 8-OH-DPAT in E, PF and C animals. Importantly, although anxiety disorders are more prevalent in females than in males (Palanza, 2001; Blanchard et al., 1995), the majority of research on

anxiety and anxiolytics has focused on males as test subjects. Therefore, this study included both females and males as test subjects. Finally, since at some doses, 8-OH-DPAT appears to enhance feeding behaviour through an increase in appetite (i.e., orexigenic effects) additional conditions and measures were added to control for this possible confound.

2. Methods

2.1. Prenatal ethanol exposure

Sprague–Dawley female (200–250 g) and male rats (250–275 g) were obtained from the Animal Care Centre, University of British Columbia, Vancouver, B.C., Canada. A 12:12 h light-dark cycle was maintained in the colony room, with lights on from 0600 to 1800 hours. Animal room temperature was maintained at 21 °C. Females were bred in hanging stainless steel cages ($25 \times 18 \times 18$ cm), with mesh front and floor. During mating, animals were allowed ad libitum access to standard laboratory chow (Jamieson's Pet Food Distributors Ltd., Delta, B.C.) and water. On the first day of gestation, determined by the presence of a vaginal plug, female rats were rehoused in polycarbonate cages ($24 \times 16 \times 46$ cm) and randomly assigned to one of three prenatal treatment groups: 1) Ethanol-fed (E) females received a liquid diet containing 36% ethanol-derived calories; 2) Pair-fed (PF) females received a liquid diet identical in nutrients and calories to that of E females, with maltose-dextrin substituted for ethanol, in an amount matched to that consumed by an E female, per kilogram body weight per day of gestation; 3) Ad libitum-fed control (C) females received unrestricted access to standard laboratory chow and water. Liquid ethanol and control diets were formulated to meet the nutritional requirements of pregnant dams (Weinberg and Bezio, 1987) and were prepared by Bio-Serv, Inc (Frenchtown, NJ). Animals were maintained on experimental diets until gestation day (d) 21. Diet bottles were weighed and replaced daily at approximately 1600 hours. As the corticoid rhythm in animals receiving a reduced ration, such as PF animals, entrains to the time of feeding, we fed animals within 2 h of lights off, permitting maintenance of a normal corticoid rhythm (Gallo and Weinberg 1981). All animal use procedures were in accordance with the Canadian Council on Animal Care and the NIH Guidelines for the Care and Use of Animals, and were approved by the University of British Columbia Animal Care Committee.

2.2. Preweaning and weaning treatments

On the day of birth, designated postnatal d 1, dam and pups were weighed and litters culled to 5 females and 5 males. Litters remained with their natural mothers until weaning. Previous research indicates that cross-fostering is not an essential procedure in our animal model, as ethanol-induced alterations in mother-pup interactions appear to result primarily from effects on the pup (Ness and Franchina 1990) rather than through alterations in maternal behavior (Vorhees 1989). Dams and pups remained undisturbed except for weighing and cage changing on gestation days 1, 7, 14, and 21 and postnatal days 1, 8, 15, and 22.

2.3. Animals

Animals were housed under a normal light cycle with lights on from 0600 to 1800 hours. On postnatal d 22 pups were weaned and re-housed with same sex littermates until 2 days prior to the start of the habituation period, when animals were singly housed for the duration of the experiment. Habituation to the graham crumbs began when the animals were approximately 90–120 days of age. In each testing condition, 12 animals of each sex, from each prenatal treatment condition were utilized.

2.4. Drugs

8-OH-DPAT was obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). 8-OH-DPAT (0.06 mg/kg) or vehicle (0.9% NaCl) was administered. The dosage for 8-OH-DPAT was determined through pilot studies in our laboratory. 8-OH-DPAT was dissolved in vehicle on the day of usage and injected subcutaneously (dorsal flank) at a volume of 1 ml/kg while rats were loosely restrained.

2.5. Novelty-induced suppression of feeding

2.5.1. Habituation

Animals were habituated to a novel, palatable food (No Name™ graham crumbs) in their home cages daily, for 15 min/day, for 21 days. Each day, approximately 4 h after lights on, the rat was gently removed from the cage by the tail and a heavy, clear glass dish with angled sides (Anchor Hocking glassware, 10.5 cm top, 6.5 cm base, 4 cm height) containing 10 g of the graham crumbs was placed into the centre of the cage before the rat was returned to the cage. The dish was removed from the cage after 15 min. Animals were food deprived for 24 h prior to the first habituation day to facilitate consumption of the graham crumbs. Chow was replaced after this initial deprivation and exposure to the food, and rats were maintained on ad libitum access to chow for the remainder of the habituation period. Animals were weighed on days 7, 14 and 21 of habituation and the amount of graham crumbs consumed was measured at the end of each habituation period on these days.

2.5.2. Baseline-feeding behaviour

To determine baseline-feeding behaviour with minimal disturbance, animals were observed in their home cages in the colony room on the last day of habituation (d 21). Variables measured included latency to begin feeding (min), duration of feeding (min), number of feeding bouts, and amount consumed (g/100 g body weight). From these variables, two additional variables were calculated; amount consumed per minute (g/min) (i.e., feeding rate) and amount consumed per feeding bout (g/bout). A feeding bout was defined as a continuous period of feeding temporally separated from any other activity by 10 s or

more, and the number of bouts was totaled at the end of the 15-min exposure period. Animals were observed and behaviour recorded in real time. Interrater reliability for all the above variables was high ($r=0.97$).

2.5.3. Behavioural testing in novel and home environments

On the day following the habituation period (d 22), animals were randomly assigned to receive either 8-OH-DPAT or saline, and 30 min after injection were presented with the graham crumbs in either their home cage or a novel cage (new home cage without bedding). Thus animals were in one of four conditions: 1) saline treatment, test in the home cage, 2) 8-OH-DPAT treatment, test in the home cage, 3) saline treatment, test in the novel cage, or 4) 8-OH-DPAT treatment, test in the novel cage. Observers blind to prenatal treatment and drug condition again recorded the same measures taken for baseline feeding. Analysis of home cage feeding behaviour served as a control for the possible appetite enhancing effects of 8-OH-DPAT. That is, as the home cage is not expected to be anxiogenic, any drug effects observed in this condition should not be due to the anxiolytic effects of the drug, but rather, to effects of the drug on appetite. In addition, each animal's normal laboratory chow was weighed before, during and after (days 20–23) behavioural observation in the home and novel cages to provide an additional measure of drug effects on appetite, i.e., orexigenic effects. Crumbs that were spilled in the novel cage were swept up and weighed; however, spillage was usually negligible. Animals tested in the home cage were observed in real time in the colony room. Animals tested in the novel cage were observed in a room adjacent to the colony room and were observed individually, to minimize disturbances in the colony room.

2.6. Statistical analysis

Principal component factor analysis was applied as a data reduction procedure for all the behavioral variables except percent of animals feeding. Raw scores were converted to standard scores and a principal component factor analysis with varimax rotation was performed. Variables that loaded significantly were further analyzed by repeated measures analysis of variance (ANOVA) for the factors of prenatal treatment, sex, drug, and test condition (home or novel environment) and day of testing (d 21-baseline, d 22-test day). Developmental data were analyzed by ANOVAs for the factors of prenatal treatment, sex and day, as appropriate, with day as a repeated measure. Significant main and interaction effects from all analyses were further analyzed by Newman–Keuls post hoc tests.

3. Results

3.1. Developmental effects of prenatal ethanol exposure

Ethanol intake of pregnant females was consistently high throughout gestation, averaging 11.36±1.55, 12.66±1.31 and 12.28±1.17 g/kg body weight for weeks 1, 2 and 3 of gestation, respectively. Repeated measures ANOVAs revealed significant differences in dam weight gain throughout the gestation period [$F(6, 123)=4.20$, $p<0.001$]. There were no differences among prenatal groups on d 1 or 7 of gestation; however, on d 14 both E and PF dams weighed significantly less than C dams (p's<0.05). On d 21 of gestation, E dams weighed significantly less than C dams, while PF dams did not differ from either group ($p=0.01$). While there were no differences among groups in the number of live born pups, there was a significant effect of prenatal treatment on total litter weight [$F(2, 33)=4.08$, $p<0.05$]. E and PF litter weighed less than C litters (p's<0.05). There were also significant interactions between prenatal treatment and age for mean pup weights for both female [$F(6,114)=7.22$, $p<0.0001$] and male [$F(6,117)=7.09$, $p<0.0001$] pups (Table 1). There were no significant effects of prenatal treatment on pup birth weights. However, on d 14, E and PF males weighed significantly less than C males (p's<0.05), and on d 21, both E and PF females and males weighed significantly less than C females and males (p's<0.01). No weight differences existed among prenatal groups at the time of behavioural testing (postnatal days 120–150).

3.2. Control for the orexigenic effects of 8-OH-DPAT

As noted, each animal's normal chow was weighed on days 20, 21, 22 and 23, to determine whether the chosen dose of 8-OH-DPAT had orexigenic effects. Thus a comparison was made of baseline chow consumption prior to drug/vehicle treatment and in the 24-h period following drug/vehicle treatment. Neither females nor males showed an increase in amount of chow consumed after saline or 8-OH-DPAT treatment. In 8-OH-DPAT-treated females, there was actually a decrease in consumption of chow compared to basal consumption [$F(1, 118)=7.06$, $p=0.009$].

3.3. Novelty-induced suppression of feeding

3.3.1. Overall factor analysis

An overall factor analysis including all the data from days 21 and 22 revealed two factors accounting for 27% and 25% of the variance respectively. Duration, amount, and amount per bout on d 21 loaded onto Factor 1 and these same variables on d 22 loaded onto Factor 2. These results suggest that behavioural testing on d 22 did indeed evoke an affective state

Table 1
The effects of prenatal ethanol exposure on mean pup weight on postnatal (PN) days 1 and 21 (g, mean±SEM)

	PN1		PN21	
	Female	Male	Female	Male
E	5.3±0.2	5.8±0.2	*42.7±1.6	*44.9±1.8
PF	5.8±0.2	6.1±0.2	*46.2±1.7	*48.4±1.8
C	6.5±0.1	6.9±0.1	52.0±1.3	55.5±1.3

* On PN 21, E and PF females and males weighed significantly less than C females and males (E±PF<C; p's<0.01). There were 12 litters per prenatal treatment condition.

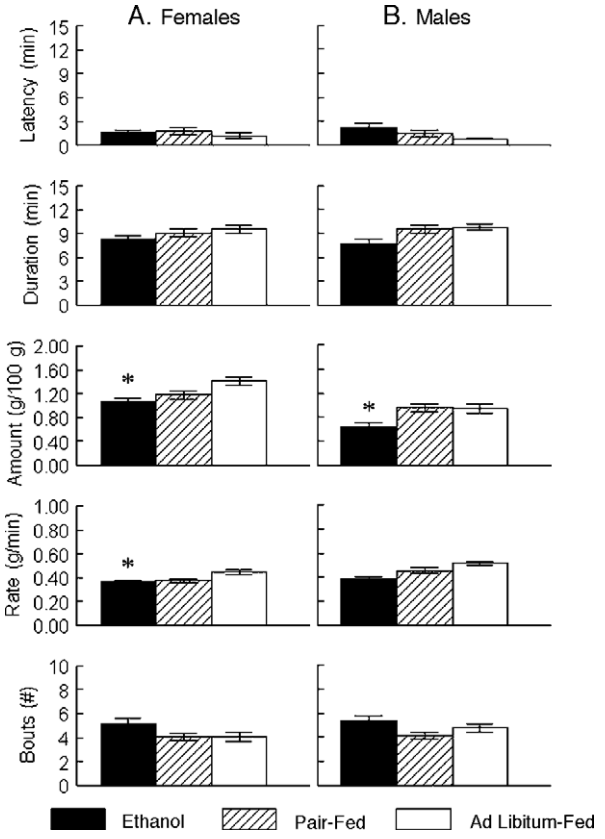

Fig. 1. Baseline-feeding behaviour on day 21 of habituation in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females and males. E females and males consumed significantly less food than C females and males (*$p<0.05$). E females also had a lower feeding rate than C females.

different from that on d 21, the final day of habituation. Factor 1 likely reflects appetitive behaviour whereas Factor 2 likely reflects anxiety-like behaviour. As we hypothesized that only the novel environment should induce anxiety on the test day, separate factor analyses were then performed for home cage and novel test conditions on d 22. Finally, to further analyse the effects of prenatal treatment and 8-OH-DPAT on the factors shown to be significant, repeated measures of ANOVAs for prenatal treatment (E, PF, C), drug (vehicle, 8-OH-DPAT) and day (d 21 and d 22), with day as a repeated measure, were performed for females and males separately for each of the variables loading onto the factors in the home and the novel test conditions.

3.3.2. Analysis of significant variables loading onto factors on d 22 (test day)

3.3.2.1. Home cage condition. Two factors emerged for animals tested in the home cage. Factor 1, likely reflecting appetitive behaviour as in the overall analysis, consisted of duration, amount and amount per bout, and accounted for 43% of the variance, while Factor 2, possibly reflecting feeding activity or general activation, consisted of number of bouts and feeding rate, and accounted for 30% of the variance.

For females, analysis of the variables loading onto Factor 1 indicated that amount consumed and amount consumed per bout were not significantly affected by either prenatal ethanol exposure or drug treatment. The finding that there were no effects of 8-OH-DPAT suggests, as expected, that this drug was not orexigenic and not anxiolytic for these variables in the home cage condition. In contrast, there was a significant interaction between drug and day for duration of feeding [$F(1,67)=4.26$, $p=0.04$] (Figs. 1 and 2). Duration of feeding decreased from d 21 to d 22 ($p<0.005$) in females receiving vehicle on d 22, but remained the same from d 21 to d 22 in females receiving 8-OH-DPAT, indicating an anxiolytic effect of 8-OH-DPAT. For the variables loading onto Factor 2, there were no significant effects of prenatal ethanol exposure or 8-OH-DPAT treatment on number of feeding bouts on d 22 (Fig. 2). However, there were significant main effects of prenatal group [$F(2,66)=3.24$, $p=0.05$] and day [$F(1,66)=4.96$, $p<0.05$] for feeding rate (Figs. 1 and 2). Post hoc analysis revealed that overall feeding rate was slower in E than in C females ($p<0.05$). In addition, feeding rate was significantly faster on d 22 than on d 21.

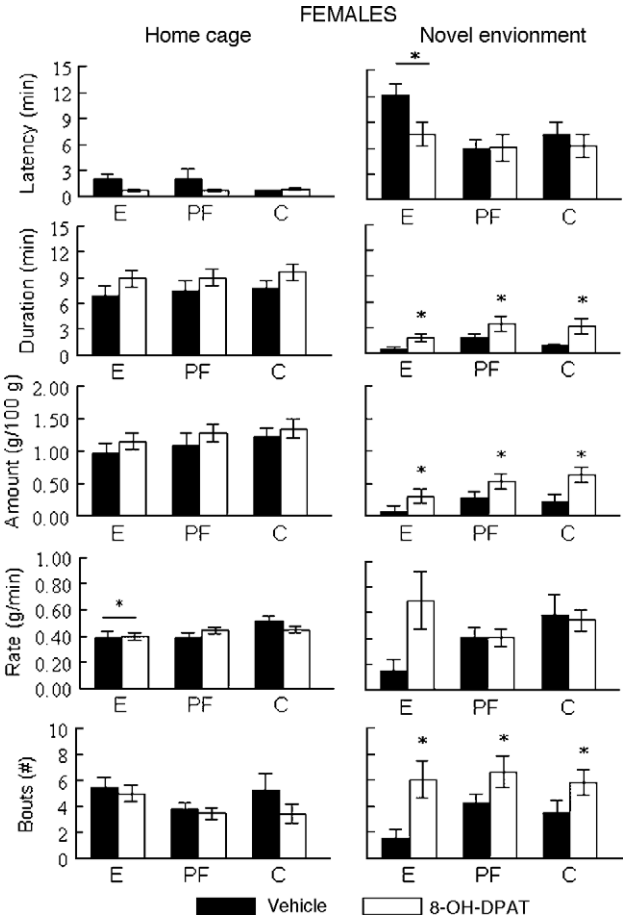

Fig. 2. Feeding behaviour in the home and novel cage conditions in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) females following vehicle (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg) treatment. In the home cage, E females had a slower rate of feeding than C females (*$p<0.05$). In the novel environment, E females had a significantly longer latency to begin feeding than PF and C females (*$p<0.01$). In addition, drug treatment significantly increased duration of feeding (*$p<0.05$), amount consumed (*$p<0.05$) and number of feeding bouts (*$p<0.0005$).

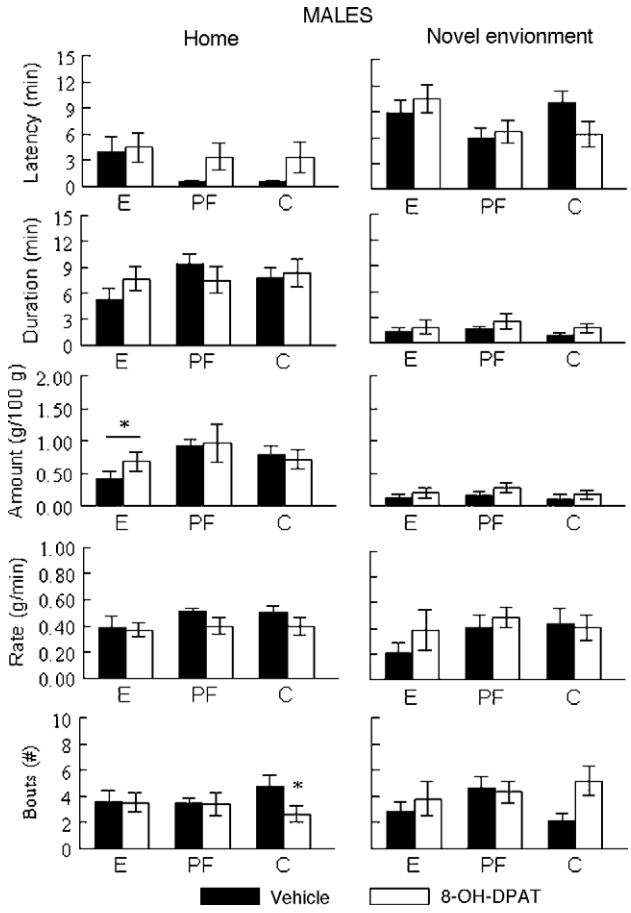

Fig. 3. Feeding behaviour in the home and novel cage conditions in ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) males following vehicle (1 ml/kg) or 8-OH-DPAT (0.06 mg/kg) treatment. In the home cage, E consumed less than PF males (*, $P<0.05$). No effects of prenatal treatment condition or drug treatment were observed in the novel environment.

For males (Figs. 1 and 3), in contrast to females, among the variables loading onto Factor 1, there were significant main effects of prenatal group [$F(2,64)=3.43$, $p<0.05$] and day [$F(1,64)=9.51$, $p<0.005$] for amount consumed, and a significant interaction between day and drug treatment [$F(1,55)=5.09$, $p<0.05$] for amount per bout. Over days 21 and 22, E males consumed less than PF males ($p<0.05$), and marginally less than C males ($p<0.096$), and overall, the amount consumed on d 22 was less than on d 21 ($p<0.005$). In addition, 8-OH-DPAT treatment resulted in increased consumption per feeding bout (g/bout) from d 21 to d 22 ($p<0.05$), whereas there was no change in this variable from d 21 to d 22 in males treated with vehicle on d 22, again reflecting the anxiolytic effect of 8-OH-DPAT (data not shown). Among the variables loading onto Factor 2, there was a significant interaction among prenatal group, drug and day [$F(2,64)=3.30$, $p<0.05$] for the number of feeding bouts; 8-OH-DPAT treatment resulted in a decrease in the number of bouts from d 21 to d 22 in C, but not E or PF males ($p=0.01$).

3.3.2.2. Novel cage condition. In contrast to the home cage condition, only one factor emerged for animals tested in the novel cage on d 22, consisting of duration, amount, and amount per bout (similar to what was found for home cage testing), as well as latency and number of bouts, and accounting for 71% of the variance. Since only one factor emerged in the novel environment accounting for a large percentage of variance, this factor is most likely a reflection of the anxiety evoked by the novel test condition. The finding that latency to begin feeding loaded significantly only onto this factor, suggests that this variable is unique to the novel environment and may be a particularly sensitive measure of anxiety-like behaviour.

For females, in contrast to the home cage condition, testing in the novel cage revealed a number of significant effects of prenatal ethanol exposure and 8-OH-DPAT treatment among the variables loading onto the one significant factor. Significant interactions between prenatal group and day were observed for latency to begin feeding [$F(2,63)=9.04$, $p<0.001$] and amount consumed (g/100g body weight) [$F(2,59)=3.59$, $p<0.05$] (Figs. 1 and 2). While there were no significant differences in latency among E, PF and C females on d 21, E females had significantly longer latencies to begin feeding compared to both PF and C females on d 22 (p's<0.05). Inspection of Fig. 2 reveals that this effect was driven primarily by a significant increase in latency in the saline-treated animals. Furthermore, while E females consumed less than C females on d 21 (Fig. 1), differences among groups were no longer significant during testing in the novel cage on d 22 (Fig. 2). In addition, day and drug interactions were observed for latency [$F(1,63)=7.69$, $p<0.01$], amount consumed [$F(1,59)=10.11$, $p<0.005$], duration of feeding [$F(1,63)=9.35$, $p<0.005$] and number of feeding bouts [$F(1,63)=17.90$, $p<0.001$]. Although females overall showed an increased latency, as well as decreased amount and duration of feeding from d 21 to d 22 (p's<0.005) those treated with 8-OH-DPAT on d 22 had marginally shorter latencies ($p<0.06$), consumed more ($p<0.05$), had more feeding bouts ($p<0.0005$), and fed for a longer duration ($p<0.05$) than those treated with vehicle (Figs. 1 and 2). Together, these results support the factor analysis, indicating that the novel environment is anxiogenic, and that 8-OH-DPAT is acting as a strong anxiolytic agent in the novel environment.

For males, among the variables loading onto the single factor, there was a significant interaction between prenatal group and day [$F(2,61)=4.96$, $p=0.01$] for amount consumed. E males consumed less than C males ($p<0.05$) on d 21 (Fig. 1). However, all males decreased their intake from d 21 to d 22 (p's$=0.001$), and differences among prenatal treatment groups were no longer significant on d 22 (Figs. 1 and 3). In addition, there were significant main effects of day for latency to begin feeding [$F(1,63)=90.10$, $p<0.05$] duration [$F(1,43)=44.7$, $p<0.0001$], bouts [$F(1,63)=286.6$, $p<0.0001$] and amount per bout [$F(1,43)=44.70$, $p<0.0001$]. Latency increased and duration, bouts and amount per bout decreased from d 21 to d 22 (Figs. 1 and 3; amount per bout not shown).

3.3.3. Percentage of animals feeding in the home and novel cage conditions

As the percent of animals feeding was a qualitatively different variable from those discussed above, it was not included in the factor analysis. ANOVAs revealed significant

Table 2
Percent (%) of ethanol-fed (E), pair-fed (PF) and ad libitum-fed (C) animals feeding in the home cage on day 21 (baseline) and in the novel environment on day 22 following vehicle (1 ml/kg 0.9% NaCl) or 8-OH-DPAT (0.06 mg/kg)

	Baseline feeding day 21 (home cage)		*Novel environment day 22*			
			Females (%)		Males (%)	
	Females (%)	Males (%)	Saline	8-OH-DP	Saline	8-OH-DP
E	96	93	33	89	50	50
PF	98	98	83	78	70	80
C	98	100	75	100	70	70

There was a significant effect of prenatal treatment condition and drug. There were significantly fewer E females feeding in the novel environment compared to PF and C females (p's<0.05). In addition, 8-OH-DPAT treatment resulted in an increase in the percent of females feeding in the novel environment, in comparison to saline treatment ($n=12$ per group). There were no significant effects among males.

main effects of prenatal group [$F(2,63)=3.58$, $p=0.03$] and drug [$F(1,63)=6.27$, $p=0.01$] (Table 2). There were significantly fewer E than PF and C females feeding in the novel environment (p's<0.05). In addition, regardless of prenatal treatment, there were fewer saline-treated than 8-OH-DPAT-treated females feeding in the novel environment. In support of these findings, the interaction between prenatal treatment and drug approached significance ($p=0.08$), suggesting that following saline treatment, fewer E than PF and C females fed in the novel environment, and this difference was eliminated by 8-OH-DPAT treatment. There were no significant effects of prenatal group or drug on the percent of males feeding in the novel environment.

4. Discussion

The present study utilized an adaptation of the novelty-induced suppression of feeding task to assess anxiety-like behaviour and the anxiolytic effect of 8-OH-DPAT in animals prenatally exposed to ethanol. There were several important findings in this study. The data support and extend previous studies (Merali et al., 2003; Muller-Gass et al., 2000) demonstrating that this anxiety-induced suppression of feeding task is an effective, simple task for assessing anxiety-like behaviour and the effects of anxiolytic agents. The novel test environment was indeed anxiogenic, and 8-OH-DPAT acted as a strong anxiolytic agent, although overall, effects were greater in females than in males. This drug had no orexigenic effects overall, and as expected, no major anxiolytic effects were observed in the home cage test condition. It is likely that injection was a mild stressor, thus accounting for the observed anxiolytic effects of 8-OH-DPAT in the home cage. Importantly, prenatal ethanol exposure altered several aspects of behavioural responsiveness in this task, both during habituation to the novel palatable food, and following testing in both the home and novel environment.

The overall factor analysis revealed that the d 21 variables duration, amount and amount/bout loaded onto Factor 1 and these same variables on day 22 loaded onto Factor 2. The finding that d 21 and d 22 variables loaded separately onto Factor 1 and Factor 2 suggests that feeding behaviour on day 21 and 22 are indeed reflective of two different affective states. These results demonstrate that these three variables are sensitive to affective state, and load together both when animals are habituated and tested under low stress conditions in their home cage or exposed to a higher stress novel testing condition. Separate factor analyses on home and novel cage variables for d 22 revealed that, in contrast to the home cage condition, only one factor emerged for animals tested in the novel cage on d 22, and that latency to begin feeding uniquely loaded onto this single factor. These results suggest that this single factor likely reflects the anxiety evoked by the novel test condition. Furthermore, latency to begin feeding may be an especially sensitive indicator of anxiety-like behaviours, and may reflect hyponeophagia.

Further ANOVAs performed on the variables loading significantly onto the factors that emerged support and extend the results of the factor analyses indicating that the novel environment was anxiogenic for both females and males, and that 8-OH-DPAT acted as an anxiolytic agent. Both females and males showed increased latency to feed as well as decreased duration of feeding from d 21 to d 22. In addition, amount consumed decreased for females, and number of bouts and amount per bout decreased for males. Interestingly, however, 8-OH-DPAT had anxiolytic effects primarily in females, resulting in a marginal decrease in latency to feed, and significant increases in amount consumed, duration of feeding and number of bouts. These data support and extend the conclusions of Muller-Gass et al. (2000) that this anxiety-induced suppression of feeding task is an effective, simple task to measure anxiety-like behaviour and anxiolytic drug effects.

Prenatal ethanol exposure altered several aspects of behavioural responsiveness in this task. Amount consumed was lower in both E females and males than in their control counterparts on d 21, the final day of habituation, suggesting a possible delay or deficit in response habituation to novel or repeated stimuli. This explanation is consistent with data from previous studies demonstrating deficits in response habituation to an olfactory stimulus in preweanling E rats (Hunt and Phillips, 2004), and in the corticosterone and adrenocorticotropin responses to repeated restraint stress in E adults (Weinberg et al., 1996). Importantly, deficits in response habituation are implicated in disturbances in memory and cognitive function (Hunt and Phillips, 2004). During home cage testing on d 22, overall feeding rate was slower in E than in C females, and E males consumed less than PF and C males. In addition, a smaller percentage of E than PF and C females fed in the novel environment, and latency to feed, one of the variables most sensitive for revealing anxiogenic effects of novel cage testing, was significantly increased in E females but not males, compared to their control counterparts. These findings indicate that prenatal ethanol exposure results in increased anxiety-like behaviour in adulthood, with greater effects in females than in males, at least as measured in this task. It is noteworthy that this increase in anxiety-like behaviour in E animals was demonstrated in a task relatively free of locomotor and exploratory confounds. Further, the data suggest that prenatal ethanol-induced hyponeophagia is, in part, mediated by the 5-HT_{1A} receptor.

There are well-known sex differences in the serotonergic system and our laboratory and others have previously found sex differences in functioning of the hypothalamic–pituitary–adrenal (HPA) axis (Kim et al., 1999a,b; Lee and Rivier, 1996; Taylor et al., 1982; Weinberg, 1988, 1992; Weinberg et al., 1996; Zhang et al., 2005). As serotonin plays a key role in modulating and regulating the HPA axis and in turn, the HPA hormones modulate serotonergic function, an alteration in the bidirectional interactions between the HPA axis and the serotonergic system may play a role in the differential effects of prenatal ethanol exposure on anxiety-like behaviours in E males and females.

Percent of animals feeding in the novel environment appears to provide one of the strongest indicators of novelty-induced suppression of feeding. Others have also used this variable as a measure of hyponeophagia (Caldji et al., 2000). We found a marked decrease in the percent of E compared to PF and C females feeding in the novel environment. Furthermore, in E females but not males, 8-OH-DPAT treatment increased the percent of animals feeding to levels comparable to those of PF and C animals. Our work has previously reported that both E females and males show greater decreases in levels of anxiety than controls when administered the benzodiazepine, diazepam (Osborn et al., 1998b). Benzodiazepines increase whole brain levels of 5-HT and its metabolite, 5-hydroxyindoleacetic acid. The present finding of an 8-OH-DPAT-induced increase in percent of animals feeding may thus indirectly implicate an altered serotonergic system in E animals (Chase et al., 1970). We have also found that E females have increased levels of CORT compared to controls following testing on the elevated plus-maze (Osborn et al., 1998a), a widely used task for assessing anxiety-like behaviours. Recent research in normal rodents has found that adrenalectomy results in decreased responsiveness to ipsapirone and buspirone, anxiolytics that exert their actions through the 5-HT_{1A} receptor (Lopez-Rubalcava et al., 1999). This suggests that glucocorticoids may contribute to the anxiolytic effects of serotonergic agonists. It is possible that since E animals respond to stressors with increased levels of corticosterone (Kim et al., 1996, 1999a,b; Lee et al., 2000; Osborn et al., 1998a; Weinberg et al., 1996) HPA hyperresponsiveness may play a role in mediating differential responses to certain drugs in E animals.

Interestingly, there was a large sex difference in percent of animals feeding in the novel environment. Suppression of feeding was similar for vehicle-treated females and males, a result in agreement with other tasks inducing hyponeophagia (Shephard and Broadhurst, 1982). In contrast, a strong anxiolytic effect of 8-OH-DPAT was observed primarily in females. These data clearly indicate the importance of including females in experiments utilizing animal tests of anxiolytic drug effects. Analysis of other feeding behaviours in this study also support the conclusion that females exhibit a greater sensitivity to 8-OH-DPAT compared to males, a finding also reported by Blanchard et al. (1992). Sex differences in functioning of the serotonergic system are well documented and contribute to numerous behaviours including anxiety. The dorsal raphe nucleus may be one of the major brain region mediating these sex differences. For example, Dominguez et al. (2003) have found that serotonergic turnover in the DRN was greater in females than in males.

Prenatal ethanol-induced alterations in the serotonergic system have important consequences for the offspring, as serotonin is involved in many behavioural and physiological functions. The neurotrophic actions of 5-HT are exerted through 5-HT_{1A} receptors. Activation of the 5-HT_{1A} receptor results in growth cone elongation, neurite outgrowth and release of the growth factor S100ß from glial cells (Azmitia, 2001). Previous studies have shown that prenatal ethanol exposure results in a decrease in fetal 5-HT (Druse et al., 1991) and subsequently results in a decrease in 5-HT_{1A} stimulation and therefore abnormal development of the 5-HT system. In addition, in normal rats, there is a developmental increase in central 5-HT_{1A} receptors from postnatal days 19 to 35. In E rats, this developmental increase in 5-HT_{1A} receptors does not occur in the septum and cortex (Kim et al., 1997). Developmental alterations in 5-HT_{1A} receptors may be an important mechanism underlying the increased anxiety observed in E animals. For example, Gross et al. (2002) have recently reported on the contribution of postnatal 5-HT_{1A} receptors in the septum and cortex in expression of normal anxiety-like behaviour in mice. E animals also exhibit a reduction in 5-HT_{1A} receptor binding (Tajuddin and Druse, 1989b) and 5-HT innervation of the frontal cortex (Zhou et al., 2002). In addition, it has recently been suggested that moderate 5-HT depletion with neurotoxins can be anxiogenic (Green and McGregor, 2002). Decreased 5-HT levels in E offspring have been well documented (Clausing et al., 1996; Druse et al., 1991; Elis et al., 1976). Therefore, early prenatal ethanol-induced alterations in 5-HT and the 5-HT_{1A} receptor in E animals may contribute to expression of anxiety-like behaviour later in life.

Finally, we found that some changes in behaviour observed in the present study may be mediated by nutritional effects associated with maternal consumption of ethanol. E and PF females and males showed a delay in weight gain during the preweaning period. However, no weight differences were observed at the time of behavioural testing, indicating that catch-up growth can attenuate this early life deficit. In addition, E females did not differ significantly from PF females in feeding rate both on d 21 and during home cage testing on d 22, or in amount consumed on d 21. It is not uncommon in the literature to find that some of ethanol's adverse effects are nutritionally mediated, as ethanol has marked effects on many aspects of nutrition.

In summary, the present data demonstrate that the novelty-induced suppression of feeding task is a simple task with many advantages over traditional tests of anxiety. The small size of the testing environment (i.e., standard housing cage without bedding) allows assessment of anxiety-like behaviour independent of the possible confounding effects of exploration or activity, and behavioural testing in the home cage can serve as a control for extraneous drug effects. In addition this task does not require food deprivation or extensive or repeated handling. We found that all animals exhibited a suppression of feeding in

the novel environment in comparison to baseline feeding levels in the home cage, as evidenced by increases in latency to begin feeding, and decreases in feeding duration and food consumption. Although an 8-OH-DPAT-induced increase in feeding behaviour is the index of anxiety levels during testing in the novel cage, the effect observed was not due to orexigenic effects of the drug. Furthermore, this task was sensitive in revealing prenatal ethanol-induced increases in anxiety-like behaviour in adulthood. Thus, our adaptation of this novelty-induced suppression of feeding task demonstrates anxiogenic behaviours, is sensitive to the 5-HT_{1A} anxiolytic 8-OH-DPAT, can elucidate effects of prenatal treatments and can serve as a test of anxiety-like behaviour in female and males rodents in the absence of some of the confounds present in other commonly used tests.

An experimental model of the affective disturbances observed in individuals affected with FASD has great clinical relevance. In addition, serotonergic anxiolytics are highly prescribed in the treatment of anxiety disorders. Therapeutic efficacy of these drugs may depend on their ability to alter 5-HT release, metabolism, reuptake or receptor function, alterations that may be affected by prenatal ethanol exposure. Therefore, an understanding of 5-HT function in an animal model of prenatal ethanol exposure may facilitate treatment of this affected population.

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