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Sex differences in emotionality in C3H/HeH mice, with hypogonadal mutant to distinguish activational effects of gonadal hormones

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ABSTRACT

The C3H/HeH mouse strain has a mutant hypogonadal (hpg) variant, providing an animal model to examine the activational effects of sex hormones because reproductive maturation is arrested at a neonatal stage. Thus in the adult mouse, the circulating concentrations of sex steroids are extremely low. The present study used a series of tests to distinguish sex differences in behaviour: open field, locomotor activity, hyponeophagia, and novel location recognition. The results showed some evidence for a role of sex hormones in emotionality underscoring the potential utility of the hpg model, to distinguish activational effects in the C3H/HeH strain. However, the direction that the sex differences took varied by task: whilst males showed the predicted sex difference of relatively greater anxiety in the open field, hyponeophagia tests suggested higher emotionality in females. The hpg mice of both sexes showed a reduction in anxiety measured as hyponeophagia. Overall it can be concluded that this set of experiments supports the potential of the hpg model to investigate hormonal influences on emotionality.

1. Introduction

Sex related differences in behaviour are influenced by a variety of biological factors, most directly by sexual dimorphisms in the structure of the brain. For example, the sexually dimorphic nucleus is a collection of cells located in the preoptic area of the hypothalamus. This cluster of cells, in particular the suprachiasmatic region, contains nearly double the number of cells in adult men in comparison to adult women [1]. The same male–female difference is found in rats [2,3]. Similarly, the hippocampus is typically larger in the male than in female mammals [4,5].

Unsurprisingly, therefore, sex differences have been reported across a range of behaviours [6–18]. Such reported sex differences are controversial because results are variable and there is clear evidence of strain as well as species differences in behaviour [19–23]. However, these apparent inconsistencies may find some explanation in terms of the underlying mechanisms for sex differences. For example, there is evidence to suggest that brain dimorphisms (hypothalamic structures) in mice are different from rats [24]. Moreover, sex hormones have activational effects subsequent to their initial effects on brain organization. The activational effects of the gonadal sex hormones are also a likely mediator of biologically based differences that may contribute to the observed behavioural variability [15,25–27].

The C3H/HeH strain of mouse has a mutant hypogonadal (hpg) variant in which a truncation in the gonadotrophin releasing hormone (GnRH) gene prevents the production of GnRH [28,29]. Low levels of sex hormones are produced in late fetal and early neonatal development, and their early organizational effects mean that these mice can be differentiated as males or females on the basis of the external genitalia. However, as these mice are congenitally lacking in GnRH and thus secretion of gonadotrophins, maturation of the testes and ovaries is arrested at a neonatal stage, and there are no postnatal increases in sex steroid production [29,30]. Therefore, such mutants have only been exposed to the organizational effects of sex hormones and the later activational effects of sex hormones are eliminated. This model has the advantage that there is no need for endocrine, pharmacological or surgical intervention prior to experimentation in order to examine the activational effects of gonadal sex hormones on behaviour.

To test how sex differences are moderated by the activational effects of gonadal hormones in this model we needed to establish the basic pattern of sex differences in the wild type strain of C3H/HeH mice. This was the main aim of the present study: to test for sex differences in the wild type. At the same time, the available hpg litter mates underwent the same series of behavioural tests, to give some preliminary indication of the role that gonadal hormones might play in determining any such sex differences. The first test was the open field, a large arena with a bright overhead light causing the arena to be divided into a relatively darker outer arena and a brightly lit inner arena. This is a classic test of exploratory activity particularly likely to detect fear-related behaviours [31]. Time spent in the inner zone is held to be an indicator of reduced anxiety. Open field measures were

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followed by a test of general locomotor activity, in a more enclosed environment (provided by a modified version of the same apparatus) and under lower light levels, to reduce anxiety. Hyponeophagia tests were used to examine whether the mice showed differences in anxiety towards novel foods presented in a novel environment [10]. Thirdly, reactions to novelty were measured using novel location exploration tests [14]. This discrimination test has a cognitive component in that it relies on spatial processing and working memory to show an effect of familiarity. Weight differences by sex and genotype were analysed and taken into account statistically in the behavioural analyses because levels of testosterone can affect weight and hence activity levels [32].

2. Methods

2.1. Animals

All animal procedures were approved by the University of Nottingham Local Ethical Review Committee and were carried out in accordance with the Animals Scientific Procedures Act (UK) 1986. 14 male mice (10 wild type and 4 hpg) and 15 female mice (10 wild type and 5 hpg), aged approximately 3–5 months at the beginning of experimental testing, were used. The mice had weights ranging from 21–44 g during the months of experimentation. The cohort of mice (wild type and hpg litter mates) was derived from the laboratory-bred C3H/HeH strain. These were supplied from a breeding colony derived from stock purchased from Jackson Immuno Research Laboratories, Inc., Bar Harbor, ME. All 29 mice were used in each experiment.

Mice were housed in same-sex groups of 2–5 in plastic cages (26.5×20.0×14 cm deep), with nesting bedding and a cardboard play tube, and maintained under a 12 hour light–dark schedule (lights on at 0700 h). Temperature in the keeping room was maintained at 21±2 °C and relative humidity at 50±5%. Food and water were available *ad libitum* in the home cage. The mice were ear clipped for identification and handled regularly twice per week over a period of 4 weeks for 10 min per mouse prior to experimentation. The mice were naïve to behavioural experimentation at the start of the study. To minimise effects of stress from handling (necessary for identification) all mice were temporarily housed individually in single cages for approximately 5–15 min prior to testing. Experimental testing took place in temperature controlled rooms, also held at 22±3 °C and relative humidity at 50±5%.

Hpg mice were clearly distinguished from their wild type siblings by examination of the external genitalia. Visible differences between the mice include the shorter anogenital distance and micropenis in males and the closed or incomplete opening of the vagina in females, compared to their wild type counterparts (Fig. 1). For practical reasons, the wild type females were tested irrespective of the stage of the estrous cycle.

The light levels were maintained at a light 194 lx for open field, 6 lx for locomotor activity, 115 lx for novel location recognition, 9 lx for room 1 and 2 lx for room 2 for hyponeophagia. Movements were recorded from an overhead video tracking camera and analysed using Ethovision (Noldus Information Technology© 2001). A mouse was recorded as entering a zone once the middle of its body was tracked within the zone. Mice were weighed immediately before testing began for each experiment. All behavioural testing was conducted between 0900 and 1700 h.

2.2. Apparatus and procedure

Males and females were tested separately in an alternating sequence of normally 4 males followed 4 females, with two final groups of 2 males and 3 females, to counterbalance for time of day. There was a single exception to this: in Experiment 1B mice were tested in alternating blocks of 6–8 males and females.

2.2.1. Experiment 1A: Open field

Two open field boxes measuring 30×70×26 cm high were used to test two mice simultaneously. The boxes had transparent perspex

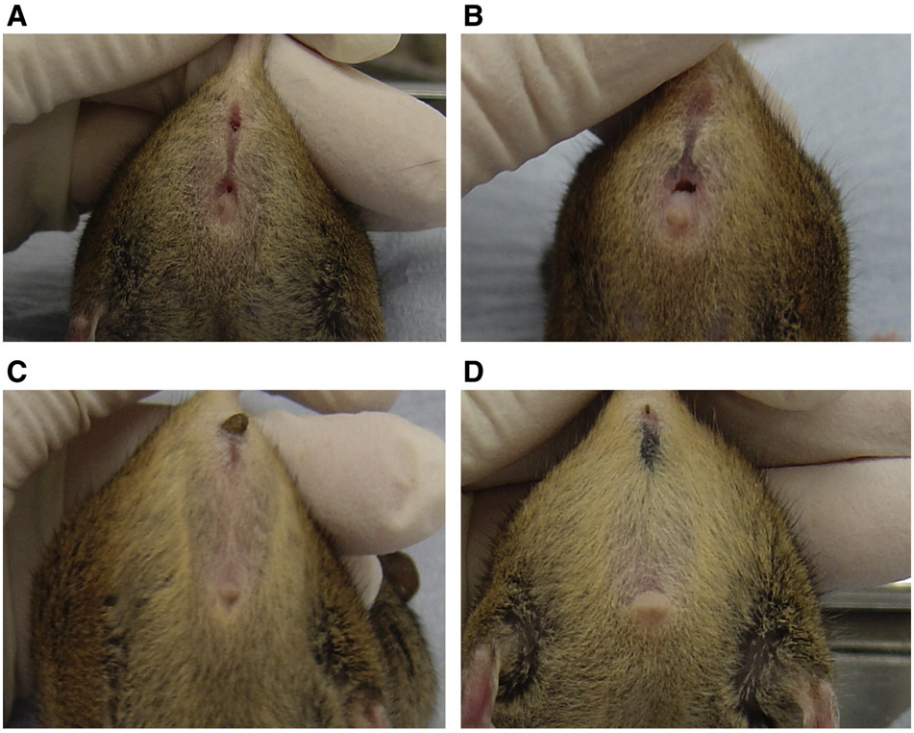

Fig. 1. A: The vagina of a hypogonadal female mouse with an incomplete opening. B: The open vagina of a wild type C3H/HeH female mouse. C: The under developed scrotum and micropenis of a hypogonadal male mouse. D: External genitalia of a wild type C3H/HeH male mouse.

walls and grey perspex flooring. The walls were blacked out using black bin liner externally and A3 white paper covered the floor so that the mice were visible for tracking. Two zones were created to measure anxiety, an outer zone (approximately 732 cm^3) and an inner zone (approximately 1353 cm^3). The dependent variables were latency to first movement (s) and the total time spent in each zone (s).

Mice were placed in the centre of the open field box and activity was recorded for 15 min. Between each test the open fields were cleaned with a 20% ethanol solution and dried off with a paper tissue. All data derive from a single exposure to the open field box and testing was completed over the course of a single day.

2.2.2. Experiment 1B: Locomotor activity

Each of the boxes used above for the open field tests was divided into four chambers by inserting 3 opaque grey perspex partitions. Each subdivided chamber measured 30×17×26 cm high. The subdivision of the boxes allowed eight mice to be tested in one session. The dependent variables were latency to first movement (s) and total distance moved (cm).

Mice were habituated to the arena 24 h prior to testing. Mice were placed separately into the centre of the divided sections of the modified open field chambers. Locomotor activity was recorded for 30 min. Between each test the apparatus was cleaned with a 20% ethanol solution and dried with a paper tissue. All data derive from a single exposure to the activity chambers and testing was completed over the course of a single day.

2.2.3. Experiment 2: Hyponeophagia

Hyponeophagia testing took place in two adjacent rooms and with two distinctive pieces of apparatus (a T-maze and a white box) to present the food, to enhance the effects of novelty. The T-maze was made out of varnished wood. The combined extent of the choice arms horizontally was 129.5 cm. The stem of the T was 80 cm. All the arm widths were 10 cm and there was a lip of 1 cm around the whole edge of the maze. The platform was raised 30 cm above the floor level. The white box (21×30×21 cm) was made out of plastic. The novel foods were chocolate chips (Supercook Milk Chocolate Chips) and sunflower seeds (Tesco Sunflower Seeds). Ten chocolate chips or sunflower seeds were placed in the same positions for each mouse. The dependent variables were time to eat (s) and number of food items eaten.

The method was the same on each of four days: ten chocolate chips or sunflower seeds were placed in the same position on the designated apparatus. The mouse was then released from the end of the T-maze, or placed in the centre of the white box, and allowed 5 min to explore the apparatus and eat the food. The experimenter recorded latency to eat the food (with a stop watch) and how many seeds or chocolate chips were eaten. At the end of the 5 min exploration time, the remaining food was disposed of and the apparatus was cleaned with 20% ethanol and dried with a paper tissue. Different combinations of testing room in and apparatus on (T-maze versus white box) which the food was presented were used to generate distinctive contexts. Each mouse was presented with both food types on each of 2 days in one of two distinctive contexts (apparatus and room) over a total of 4 days testing.

2.2.4. Experiment 3: Novel location recognition

Novel location recognition was measured in modified open field boxes (30×70×26 cm high, as in Experiment 1A). The walls were covered externally with black bin bags and the floor was covered in white A3 paper so the mice were readily detected by the tracking camera, and movements recorded onto video. The wall coverings also precluded the use of the majority of external extra maze cues.

Location recognition was tested using identical objects in each of two locations: two ceramic egg cups (4.7 cm diameter×5 cm high and hollow). During testing the objects were placed 5 cm away from the walls of the chamber to allow free exploration of the object in location. They were fixed in place with blue tack (Bostik® Blu Tack), which was changed for each trial, to eliminate odour cues.

On day 1, each mouse was placed in the centre of the box for 15 min, to allow habituation to the arena. Between each habituation session the box was cleaned with a 20% ethanol solution and dried with a paper tissue. The experimental tests were also preceded by a habituation phase in which activity was recorded for 5 min prior to the presentation of any objects. The objects were then placed in the box for 5 min exploration, held down by blue tack (Bostik® Blu Tack) 5 cm away from the walls of the box. Activity was recorded during this 5 minute sample phase. After exposure to the objects in the sample locations the mice were removed from the test arena and replaced in the holding cage. The paper in the test arena was changed and the box was wiped with 20% ethanol solution and dried with a paper tissue. This procedure took 3–4 min so this was the inter-stimulus interval (ISI). In the choice phase both identical objects were familiar from the sample phase but one such object was replaced in a familiar location and one in a novel location. Thus the task was used to measure the extent to which mice could discriminate the novel location. Object exploration at the choice phase also was recorded over 5 min. Therefore, the test procedure took a total time of up to 19 min. All data derive from a single exposure to the objects in location at test, completed over the course of a single day.

The videos were later scored for the time spent around the objects on those occasions when an object location was explored. An exploration was defined as when the mouse's nose or paws touched the object. Inadvertent approaches from the rear of the mouse were not included in the analysis of explorations. The dependent variable was exploration time (s) and the scores used for analysis were the agreed ratings of two trained experimenters.

2.3. Data analysis

Weight data were analysed by Analysis of Variance (ANOVA) and behavioural data by Analysis of Covariance (ANCOVA) in 2×2 factorial designs using SPSS software version 15.0, with between-subjects factors of sex (male or female) and genotype (wild type or hpg). ANCOVA used weight or activity levels as the covariant, as applicable. A mixed design was used where there was a repeated measures factor: experiment in the analysis of body weight over the duration of the study; familiarity to assess choice exploration of novel location – relative to sample exploration – in the location recognition analyses. Post hoc analyses were conducted using two-tailed *t*-tests.

3. Results

3.1. Weights

As would be expected, there was some increase in weight over the duration of the studies, $F(2,50)=44.86$, $p<0.001$. There was a main effect of sex, $F(1,25)=6.38$, $p<0.05$, because the males (36.16±1.20) were significantly heavier than the females (32.03±1.11). The main effect of genotype was marginal, $F(1,25)=3.14$, $p=0.09$, the hpg mice tended to be heavier (35.54±1.37) than the wild type (32.65±0.91).

3.1.1. Experiment 1A: Open field

There were no significant main effects or interactions on latency to first movement ($F_{max}(1,24)=3.46$). However, the mice spent different amounts of time in the different zones of the open field. There was a significant main effects of sex: female mice spent overall more time than male mice in the inner zone, $F(1, 24)=4.67$, $p<0.05$; male mice spent overall longer than female mice in the outer zone, $F(1, 24)=4.41$, $p<0.05$ (see Fig. 2). This pattern of effects provides evidence

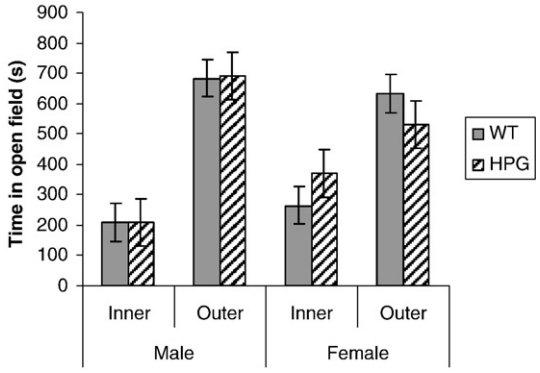

Fig. 2. Total time spent (s) in each of the open field zones, inner or outer, by sex (male or female) and genotype (wild type or hypogonadal, WT or HPG). Error bars represent the standard error of the adjusted mean for approximate between groups comparisons.

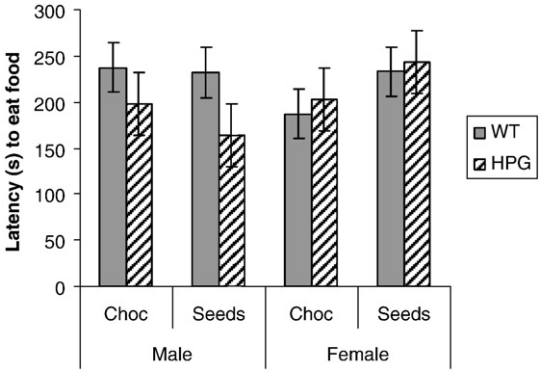

Fig. 3. Food consumption in the hyponeophagia tests, by food type, sex (male or female) and genotype (wild type or hypogonadal, WT or HPG). The dependent variable was the number of seconds taken to first eat the food. Error bars represent the standard error of the adjusted mean for approximate between groups comparisons.

that female C3H/HeH mice were overall less anxious than the males. However, there was no main effect or interaction with genotype ($F_{max}(1,24)=3.46$) and therefore no basis to conclude that this sex difference was due to the activational effects of sex hormones.

3.1.2. Experiment 1B: Locomotor activity

There were no significant main effects of sex or genotype and no significant interaction between these factors for latency to first movement or total distance moved, all $Fs<1$ (see Table 1).

3.1.3. Experiment 2: Hyponeophagia

There were no overall main effects of sex or genotype (both $Fs<1$) and no significant interaction between these factors ($F(1,24)=2.03$) on the latency to eat the novel foods. However, the latency data showed that there were sex differences in the mice' unconditioned food preferences. There was a significant interaction between sex and food, $F(1,24)=4.65$, $p<0.05$. Female mice took longer to eat the sunflower seeds compared to the chocolate chips, $t(14)=3.34$, $p=0.005$ (see Fig. 3). There was also a significant interaction between food and exposure, $F(1,24)=4.74$, $p<0.05$. This arose because mice ate overall more chocolate chips on exposure 1 (272.5±10.99) than on exposure 2 (191.81±19.63), $t(28)=6.19$, $p<0.001$. However, there were no interactions between sex, genotype and exposure, $F_{max}(1,24)=3.69$.

Consistent with the latency data, analysis of the number of food items eaten confirmed a role for sex hormones in determining the mice' unconditioned food preferences. There was again a significant interaction between sex and food, $F(1,24)=12.81$, $p<0.005$. Female mice ate more chocolate chips (2.53±1.72) compared to sunflower seeds (1.33±1.29), $t(14)=3.38$, $p=0.01$). In addition, there was a significant interaction between genotype and food, $F(1,24)=7.86$, $p<0.01$. This arose because the hpg mice ate more sunflower seeds (1.79±0.43) compared to chocolate chips (0.90±0.26), whereas wild type mice ate more chocolate chips (0.98±0.17) compared to sunflower seeds (0.67±0.28), though both of these comparisons were individually non-significant, $t(8)=1.35$ and $t(19)=1.37$, respectively. There were no overall effects of sex ($F(1,24)=0.11$) or genotype ($F(1,24)=1.97$), and no significant interaction between these factors ($F(1,24)=2.83$) on the number of food items eaten, $F_{max}(1,24)=2.05$. However, there was a significant interaction between exposure and sex, $F(1,24)=9.29$, $p<0.01$. The female mice ate overall less on the first exposure (0.67±0.72) than on the second exposure (1.27±0.88), $t(14)=2.67$, $p<0.05$ (see Fig. 4). There was also a significant interaction between exposure and genotype, $F(1,24)=15.41$, $p<0.001$. This arose because the wild type mice ate less on the first exposure (0.52±0.90) than on the second exposure (1.1±0.94), $t(19)=4.18$, $p<0.001$. Although hpg mice (males in particular) seemed to eat relatively more on the first exposure (see Fig. 4) the difference in the amount eaten on the first and second exposures by the hpg mice was not significant, $t(8)=1.38$.

3.1.4. Experiment 3: Novel location recognition

There was a significant effect of familiarity on the time spent in object location exploration during the choice phase, $F(1,24)=5.74$, $p<0.05$. The mice spent overall more time around the novel location (12.14±1.86) than the familiar location (7.94±1.98) so the novel location procedure was effective as a working memory test for the C3H/HeH mouse strain. Fig. 5 shows that the effect of familiarity was clearest in the males. However, there were no significant main effects

Table 1

	Males		Females	
	Wild type	Hypogonadal	Wild type	Hypogonadal
LMA latency (s)	1.0±1.3	2.4±1.9	2.9±1.4	1.6±1.7
LMA total distance (cm)	4630.1±596.9	5047.1±867.0	2015.4±629.7	4610.33±782.9
Hyponeophagia latency (s)	234.60±18.96	180.59±30.41	209.94±20.66	222.84±25.85
Hyponeophagia number	0.59±0.29	1.72±0.46	1.06±0.32	0.98±0.39

Legend: Means (±S.E.M) for dependent variables not illustrated elsewhere (in Figs. 2–5) or reported in the text. LMA=locomotor activity measured by latency to move and total distance covered. Hyponeophagia tests of eating in a novel environment measured as latency to eat and number of food items eaten (collapsed for food type).

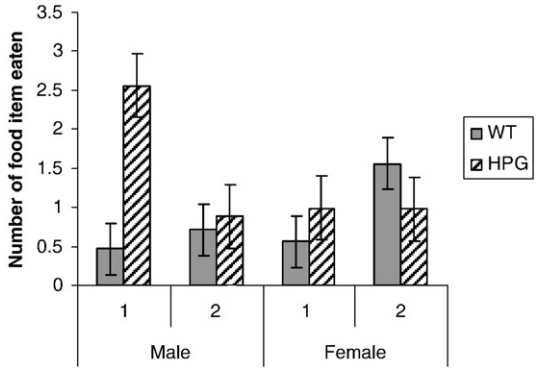

Fig. 4. Food consumption in the hyponeophagia tests, by exposure, sex (male or female) and genotype (wild type or hypogonadal, WT or HPG). The dependent variable was the number of food items eaten on each of the exposures. The legend numbers refer to the first (1) and second (2) exposures. Error bars represent the standard error of the adjusted mean for approximate between groups comparisons.

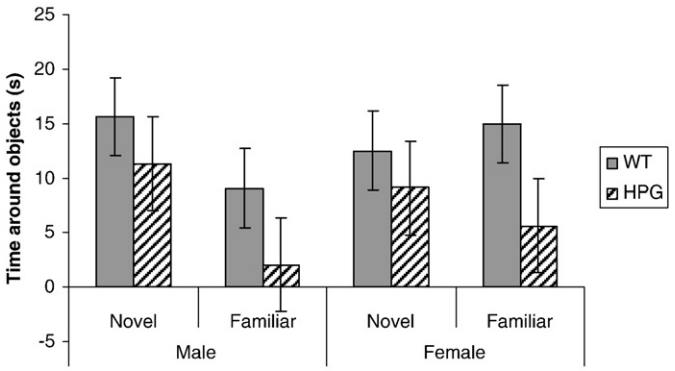

Fig. 5. Novel object location, by sex (male or female) and genotype (wild type or hypogonadal mice, WT or HPG). The dependent variable was the number of times the mouse touched or approached the object. Error bars represent the standard error of the adjusted mean for approximate between groups comparisons.

of sex ($F(1,24)=0.08$) or genotype ($F(1,24)=3.03$) and no significant interactions involving these factors ($F(1,24)=0.01$).

4. Discussion

The main aim was to establish whether there are sex differences in behaviour in C3H/HeH mice. Under conditions of low anxiety, locomotor activity tests showed no sex difference in general exploration. However, under conventional open field conditions, a sex difference emerged in that males spent more time around the edges of the arena, in the outer zone. This suggests greater anxiety in male and lower anxiety in female C3H/HeH mice, as measured by general exploration. Emotionality differences were also apparent in tests of hyponeophagia testing. Again there were differences by sex, and in this case also by genotype.

Overall the results confirmed a role for sex hormones in emotionality in the C3H/HeH mouse. On some parameters this role was apparent from the basic male female sex difference. In principle such sex differences could be attributed to the organizational or activational effects of sex hormones. The findings with respect to genotype must be regarded as preliminary in that because of the small number of hpg mice tested, there is a likelihood of type II error. Nonetheless, on some parameters, effects by genotype provided direct evidence for the activational effects of gonadal hormones in determining differences in emotionality.

4.1. Open field exploration

The fact that female C3H/HeH mice spent more time in the inner zone of the open field suggests reduced exploratory anxiety as measured in the open field. Previous studies in rats have also found females to be less anxious than their male equivalents [33–36], though demonstration of this difference can depend on the oestrus cycle [37–40]. The hpg mice used in the present study never reach sexual maturity so these females are unaffected by oestrus cycle and oestrus cycle was not measured in the wild types. However, the sex difference we observed was independent of genotype so there was no indication that such activational effects influenced performance in the open field in C3H/HeH mice.

4.2. Locomotor activity

Under conditions of lower anxiety, earlier studies have reported sex differences in general locomotor exploration in the meadow vole and mice [15,18,22]. In the present study, we found no systematic sex differences under lower lighting and in the smaller arena used in Experiment 1A. Our mice were well habituated in that the open field testing in a larger version of the arena was conducted first. The negative result on general locomotor activity reported here is consistent with others that have shown no sex difference in mice [19,41].

4.3. Hyponeophagia

This was an additional test of the mice' reaction to novelty in terms of their readiness to consume unfamiliar foods presented in novel environments. Here there were effects by both sex and genotype. Hyponeophagia is shown as a relative reluctance to eat novel foods in novel environments, particularly on the first exposure. The sex difference took the form that females took longer to eat on the first exposure. This suggests, in contrast to the open field result, relatively higher anxiety in the females. The other differences observed, for example that females took longer to eat seeds and males ate more chocolate chips cannot be used to argue for any particular direction of effects with respect to emotionality. Differences in readiness to eat different food types reflect no more than unconditioned preferences. In principle such unconditioned preferences could relate to differences in emotionality, but there is no direct evidence on this point.

The hyponeophagia test used here suggests the opposite conclusion to the open field with respect to the sex difference in emotionality. In general, there is evidence to suggest that anxiety is not a unitary phenomenon. In part the discrepancy may arise because of the engagement of different brain systems to process information about food and novel contexts [42–44]. For example, hyponeophagia, along with a number of other species-specific behaviours fundamental to emotionality, has been argued to be reliant on the normal functioning of the hippocampus [45]. Tests of anxiety that are more heavily based on exploration are not so consistently disrupted by hippocampal lesions [46,47].

With respect to genotype, wild type mice ate less on the first compared to the second exposure. This is the normal hyponeophagia effect. Although the difference was not significant, hpg mice showed a tendency toward a reversal of this pattern (males in particular, see Fig. 4). In any event they did not show normal hyponeophagia consistent with reduced anxiety in this test. However, the other effect by genotype, the overall increase in preference for sunflower seeds in hpg mice, cannot be used to argue for any particular direction of effects with respect to emotionality. The unconditioned differences in apparent food preference, by sex and genotype, are broadly consistent with sexually dimorphism in the expression of sex hormones in the hypothalamus (in zebra finch) [48]. In the present study, the unconditioned differences observed may well relate to the fact that hpg mice tend to become obese [32,49]: disinhibited feeding behaviour could be contributing factor.

4.4. Recognition of novel location

There was a basic overall effect of familiarity, so this task is suitable for testing cognitive differences in C3H/HeH mice [50]. In the present study there was no clear evidence for any role of sex hormones in determining recognition based on location. However, this should be further tested by introducing delays between the sample and test location exposures.

4.5. Non-specific confounded effects

Reduced testosterone levels are known to increase the weight of an individual, female as well as male [32]. Thus the hpg mice of both sexes would be expected to be heavier. This was no more than a non-significant tendency in the present study. However, there was a clear sex difference in weight. Throughout the study, weight or the comparable activity scores, were therefore used as covariants, to adjust for this non-specific effect.

Uterine position with respect to foetuses of the opposite sex can also result in changes in anogenital distance and later vaginal opening in females [51–54]. Whilst we cannot exclude the possibility that uterine position effects could have been confounded in the present study, the reliable hpg phenotypic differences that we observed (shown in Fig. 1) are clearly more extreme [28–30].

4.6. Compensatory effects and the hpg mutation

It is well recognised that conventional temporally constant mutations can be sub-optimal research tools to investigate basic gene function because of the likelihood that compensation over the course of development may restore or even reverse the predicted phenotype. Some aspects of sexual differentiation may be unaffected by the hpg mutation [54]. This possibility notwithstanding, the genitalia of the hpg mutant mice are clearly underdeveloped, being arrested at a neonatal stage of development, and postnatal levels of circulating sex steroids are very low [29,30]. Thus the hpg mice have clear non-compensatable hormonal deficiencies. We cannot exclude the possibility that the observed hpg phenotype could be due to compensatory changes rather than the primary genetic intervention. However, in the case of this particular mutation, the observed hormonal changes are most parsimoniously explained in terms of the truncation in the GnRH gene.

4.7. Conclusions and implications

These data provide consistent evidence for a role of sex differences in emotionality in the C3H/HeH strain. As discussed above, the direction of the sex difference in the open field and the hyponeophagia tests was different. In general, the relationship between the sex hormones and particular emotional behaviours may vary from one task to the next. Nonetheless, the role of sex hormones in emotionality in C3H/HeH mice is further confirmed by differences in the mutant mice, consistent with the utility of the hpg model in distinguishing the activational from the organizational effects of sex hormones.

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