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Male and female immediate fear reaction to white noise in a semi-natural environment: A detailed behavioural analysis of the role of sex and oestrogen receptors

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Abstract

In classical rodent anxiety models, females usually display lower anxiety than males, whereas anxiety disorders are more prevalent in women. Perhaps this contradiction is caused by the use of behavioural models with low external validity. Therefore, we analysed immediate reactions to a sudden 90-dB white noise in a semi-natural environment. We observed mixed-sex groups of rats for the 60 seconds preceding noise onset and the first 60 seconds of exposure. White noise elicited fear-specific behaviours hiding alone and huddling. It also increased exploratory and ambulatory behaviours, although only in the burrow zone farthest from the open area. Thus, in a semi-natural environment, white noise enhanced motor activity as a product of fear-induced general arousal. Then, we compared male and female sexual, social, exploratory and anxiety-related behaviour, and found little sex difference. This absence of behavioural effect, also observed in other studies, might be a result of our study design, a familiar environment with an ecologically relevant social context. Fear and anxiety responses are modulated by oestrogens through the activation of oestrogen receptors α and β. Thus, in a third part of out study, we analysed how treatment with either oil, oestradiol benzoate (EB), an agonist to the oestrogen receptor α (propylpyrazoletriol [PPT]) or β (diarylpropionitrile [DPN]) influenced female behaviour. The effect of treatment was limited, both EB and PPT stimulated motor activity in the open area before white noise, probably because of sexual activity. PPT increased the probability of fleeing from the noise, and decreased the latency to do so, which is consistent with a pattern of anxiogenic properties found in previous studies. Contrary to reports in classical procedures, we failed to detect any effect of DPN on immediate fear reactions in a semi-natural environment.

KEYWORDS

oestrogen receptors, fear, semi-natural environment, sex difference, white noise

1 | INTRODUCTION

Differences in male and female behaviour have been debated for decades, and resulted in the most exotic theories based on dubious evolutionary principles.[1] Biological factors (eg, gonadal hormones or sex chromosome genes) can partly explain at least some of the gender differences, and sex is a significant risk factor for neurodevelopmental and neurodegenerative disorders.[2] Indeed, a number of psychiatric troubles are distributed along a biased sex ratio, with these including anxiety and depression, which are more prevalent in women than in men.[3] In this context, it is highly necessary to understand how sex influences our health to improve patient care and treatment. The study of male subjects has prevailed so far, even though considerable efforts have been made to include females in the last decade. In particular, the USA National Institutes of Health recently requested to consider sex as a relevant biological variable in National Institute of Health applications.[4] However, much work remains to be done, notably to adapt the statistical methods to the investigation of sex differences and sex interaction with treatment, and to report results by sex, which is rarely achieved.[5] In the past, inadequate experimental designs, either ignoring female behaviour or focusing on passive rather than active reactions, or biased data collection have also hindered discoveries in female health.[6]

Fear and anxiety-related behaviours are usually assessed through a battery of classical tests in rodent models. The most commonly used are the open-field test, the Vogel test, the light-dark compartment test and the elevated plus-maze test.[7] These tests present a single experimental subject with a new, anxiogenic situation, allowing for quantification of behaviours, supposedly comparable across the tests. In these tests, females often show patterns of decreased fear compared to males. For example, in an elevated plus-maze, females have been reported to show more entries into the open arms, more distance travelled and less fear-related behaviours, such as freezing or defecation.[8-12] In the open field test, some data show that females cover more distance and display more rearing postures.[8,13] The proposal that females may show reduced fear in these procedures, whereas women are more at risk of developing mood disorder, is rather contradictory. However, most of the behavioural patterns collected in classical tests rely on motor activity or locomotor exploration. This ignores the fact that females sometimes display higher locomotor activity than males, regardless of the environmental context.[12,14,15] Motor activity is a potentially important confounding factor in measures of emotional status.[16] In addition, the above-mentioned classical tests suppress the social component of behaviour, despite its determining nature for highly social animals such as the rat. Indeed, social interaction has a rewarding value for rats and can induce conditioned place preference.[17] A recent review of anxiety studies in rodent models highlighted the challenge of anxiety measurements, and emphasised the need for clearer definitions of the measured variables and conditions used, to achieve greater transferability.[7] This is especially relevant to the contradiction between results obtained in female rodents and the prevalence of anxiety in women.

Brunswik et al.[18,19] defined procedures from which the results are generalisable to other contexts as procedures with an external validity. In sex difference research, anxiety studies would benefit from naturalistic conditions and complex social environments. Indeed, it has been suggested that an ethological approach could increase the translational value of animal models, particularly by incorporating group-housed animals.[20] Semi-natural environments are particularly suitable for this purpose and have already been used to study fear reactions,[21-23] as well as sexual behaviour in both sexes.[24,25] Previous studies conducted in our laboratory have looked into the expression of fear in females rats hosted in a semi-natural environment[26] and more specifically into the differential role of oestrogen receptors in emotional responses in this environment.[27,28] Indeed, the variability in male and female fear and anxiety-related behaviours is considered to rely, at least partly, on the main female hormone oestradiol.[29] This steroid modulates behaviour differently depending on the environmental context.[30] In particular, oestrogen receptor (ER)α and ERβ, present in both male and female mammals,[31,32] have different effects on fear reactions. ERα has shown anxiogenic properties in several anxiety models. A selective ERα agonist increased defecation and time spent grooming in an elevated plus-maze.[33] By comparison, reducing the expression of this receptor in the medial preoptic area alleviated indicators of fear and anxiety in the open field and the light/dark box,[34] suggesting that the activation of this receptor is anxiogenic. In parallel, when tested in a semi-natural environment, females with a reduced number of ERα in the ventromedial nucleus of the hypothalamus showed almost no huddling behaviour when exposed to aversive white noise, and they recovered fast from white noise exposure.[28] By contrast, activation of ERβ has consistently led to anxiolytic effects in the elevated plus-maze[33,35-37] and in the open field.[33] In a semi-natural environment, females treated with an ERβ agonist showed a distinct profile in response to aversive situations,[27] whereas females with a reduced number of ERβ in the central amygdala showed a pattern of increased anxiety, including increased risk assessment and decreased food consumption.[28] Thus, oestradiol plays an important role in the modulation of fear and anxiety reactions in females, through the differential activation of ERα and ERβ, which could partly explain the sex difference in anxiety prevalence. Even though there are data available showing that ER agonists modulate anxiety responses in males,[33,35] we limited the present study to an evaluation of their role in females.

Most classical anxiety tests present the experimental subject with an anxiogenic situation but not with a discrete external, fearful stimulus. To this effect, we decided to use white noise, a widely used stressor in anxiety studies. Even though it is not a standard part of the rat natural habitat, loud noise is part of the anthropogenic disturbances that can be faced by urban animals, such as the rat. Experiments previously conducted in our laboratory showed that white noise was highly aversive to the rats, efficiently eliciting classical fear- and anxiety-related behaviours.[26-28] These and other anxiety studies analysed behaviour expressed over

the entire duration of the test (ie, sustained anxiety). Immediate fear and anxiety reactions (ie, phasic anxiety) might be more informative and are worthy of special attention. There is evidence showing that phasic and sustained anxiety responses depend on different neural systems[38,39] and that they are differently modulated by drugs.[40] Because our earlier studies of the role of oestrogen receptors in fear and anxiety responses were limited to sustained anxiety, we aimed to analyse their importance in phasic anxiety. Furthermore, the fear responses of males were ignored in the earlier studies. Here, we also report data from males. Based on video recordings from a previous experiment,[27] we made a detailed ethological analysis of immediate behavioural reactions of multi-male, multi-female groups of rats housed in a semi-natural environment. Detailed analyses of the spatial distribution of behavioural activity were also made. In typical anxiety tests such as the elevated plus-maze, the dark/light choice procedure or the open field, the differential use of space is used as an indicator of fear or anxiety.[41] Therefore, we also determined the localisation of each behavioural activity in the semi-natural environment. We focused on the 60 seconds preceding the onset of a 90-dB white noise, as well as the first 60 seconds of exposure to it. Ovariectomised females were administered oestradiol or a selective ERα or ERβ agonist. Because white noise can be expected to induce fear, and since the ERα has been reported to be anxiogenic in such situations, we predicted that an ERα agonist would enhance fear reactions. Because the ERβ is generally believed to be anxiolytic, we predicted that an ERβ agonist would reduce fear responses. The effects of oestradiol, acting on both receptors, were difficult to predict. The male subjects were left intact. Indeed, there is evidence showing that conditioned fear responses are not altered by castration.[42] It may be assumed that this also is the case for unconditioned fear.

In the present study, we carefully examine noise-, sex- and treatment-effects on behaviour. The results will provide a better understanding of sex differences and the relative contribution of ERs in phasic anxiety responses, in a procedure with external validity.

2 | MATERIALS AND METHODS

2.1 | Subjects

Forty female and 30 male Wistar rats (mean ± SEM weight was 278.7 ± 2.7 g and 352.9 ± 4.8 g at the beginning of the experiment, respectively) were obtained from Charles River WIGA (Sulzfeld, Germany). Females were ovariectomised under isofluorane anaesthesia within 15 days after arrival, and 14 days prior to the beginning of the experiment, in accordance with the established surgical procedure.[43] Rats were housed in same-sex pairs in standard Makrolon® IV cages (Tecniplast, Buguggiate, Italy) from their arrival to their introduction into the semi-natural environment (ie, for approximately 30 days). During this period, water and food (RM1; Special Diets Services, Witham, Essex, UK) were available ad lib. The temperature was maintained at 21 ± 1°C and the relative humidity at 55 ± 10%. Lights were set on a reversed 12:12 hour light/dark photocycle (lights on 11.00 PM). The ventilation system in the animal facility produced an ambient noise of approximately 40 dB. All experimental procedures employed in the present experiment were approved by the Norwegian Food Safety Authority and were in agreement with the European Union council directive 2010/63/EU.

2.2 | Apparatus

The semi-natural environment used in this study has been described in detail earlier.[24,44,45] Rats typically live in burrow systems surrounded by a large area described as the home range.[46,47] To approximate the natural conditions, we provided the rats with a complex burrow system (120 × 210 cm) including several corridors and four nest boxes. Four small openings (8 × 8 cm) connected the burrow with a large open area (120 × 210 cm) furnished with three small shelters (Figure 1). The burrow was maintained in complete darkness by the use of a light-blocking wall of extruded polyethylene foam.

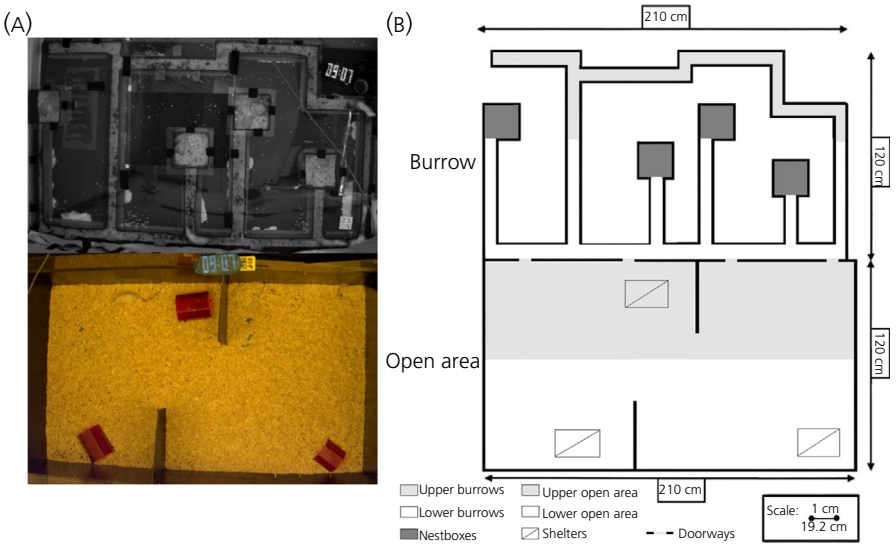

FIGURE 1 A, Picture of the semi-natural environment. B, Division in seven zones. Doorways refer to the openings connecting the burrow with the open area

TABLE 1 Summary of the experimental design

Days in the semi-natural environment	Female treatment			
	Oil	EB	PPT	DPN
Day 5	Peanut oil (Oil) 1 mL kg^{-1}	17β-oestradiol benzoate (EB) 18 μg kg^{-1}	Propylpyrazoletriol (PPT) 10 mg kg^{-1}	Diarylproprionitrile (DPN) 10 mg kg^{-1}
Day 6	Peanut oil (Oil) 1 mL kg^{-1}	Peanut oil (Oil) 1 mL kg^{-1}	Propylpyrazoletriol (PPT) 10 mg kg^{-1}	Diarylproprionitrile (DPN) 10 mg kg^{-1}
Day 7	Progesterone 1 mg per rat	Progesterone 1 mg per rat	Progesterone 1 mg per rat	Progesterone 1 mg per rat

Infrared lamps (850 nm) allowed for video recording of activity in the burrow. The open area was submitted to the same photocycle as previously noted, although a 1 lux light was maintained during the dark phase. Video recording was made possible by two cameras fixed to the ceiling, one in the burrow and one in the open area. Rats aggregate in multi-male, multi-female colonies, with a smaller proportion of male members than female ones.[46,48] Thus, groups of four females and three males were hosted in the semi-natural environment, allowing for the expression of a large range of social behaviours.

2.3 | Treatment and hormones

To evaluate the potential role of oestrogens in female fear reactions, we employed four groups of ovariectomised females: one treated with oil only (ie, no stimulation of oestrogen receptors). Another group was given EB in a dose sufficient for inducing full behavioural oestrus, simulating the oestrous phase in intact females. A third group was given an agonist selective for the ERα and a fourth group was given an agonist selective for the ERβ. In this way, we could compare females in a state similar to diestrus (oil treated group) with females in a state similar to oestrus (EB treated group), In addition, we could determine the possible role of each of the ERs. It should be noted that all groups received progesterone, which is an important part of the endocrine environment in natural oestrus. Progesterone by itself may have actions on general activity, fear responses and other behaviours.[29,49] By treating all groups with progesterone, we eliminated, or reduced, the confound between oestradiol and progesterone effects that otherwise would have occurred.

Oestradiol benzoate (EB) and progesterone (P) (both from Sigma-Aldrich, St Louis, MO, USA) were administered s.c. at a dose of 18 μg kg^{-1} and 1 mg per rat, respectively. The hormones were dissolved in peanut oil (Den norske Eterfabrikk, Oslo, Norway), with an injection volume of 1 mL kg^{-1} for EB and 0.2 mL per rat for P.

The oestrogen receptor agonists propylpyrazoletriol (PPT; ERα) and diarylpropionitrile (DPN; ERβ) were obtained from Tocris Bioscience (St Louis, MO, USA). PPT is selective to ERα, with a 410-fold preference compared to ERβ, and with a relative binding affinity of 50% compared to oestradiol.[50] DPN is selective to the ERβ, with a 72-fold preference compared to ERα, and with a relative binding affinity of 18%.[51] PPT and DPN reach their maximum serum concentration approximately 30 minutes after s.c. injection and have a half-life of 6.0 ± 0.03 hours and 8.2 ± 1.7 hours, respectively.[52] Both PPT and DPN were dissolved in undiluted dimethyl sulphoxide (DMSO; Sigma-Aldrich) right before s.c. injection, and were administered at a dose of 10 mg kg^{-1} body weight in a volume of 1 mL kg^{-1}, on two consecutive days. The rationale for using the agonists at these doses is elsewhere.27 The injection did not cause any significant necrosis at the injection site. The acute toxicity of DMSO has been reported to be low,[53,54] and adverse effects are found only at doses far superior to the amount administered here. Undiluted DMSO was also used in an earlier study on the effects of ER agonists on sexual behaviours, and no difference between DMSO and sesame oil vehicle was reported.[55] Therefore, we did not consider it justified to add an additional vehicle group to control for unlikely effects of DMSO.

2.4 | Procedure

The floor of the semi-natural environment was disinfected and covered with wood chips (Tapvei, Paekna, Estonia) prior to the experiment. The nest boxes were provided with nest material and the open area with 12 wood sticks, as well as approximately 3 kg of regular food pellets and four 0.5-L bottles of water in a corner. Rats were identified by different combinations of shaving patterns on the back associated with black marks on the tail. More details are provided elsewhere.[27]

In a previous study[27], we focused on sustained effects of emotional stimuli without analysing immediate effects of said stimuli. Here, we observed behaviour during the 1 minute preceding white noise onset and the first 1 minute of noise exposure. The noise was produced by a white noise generator (Lafayette Instruments, Lafayette, IN, USA) connected to two loudspeakers (Scan-Speak Discovery 10F/8414G10; Hifi Kit Electronic, Stockholm, Sweden), one suspended approximately 2 m above the burrow and another at the same height above the open area, producing 90-dB white noise as measured on the floor.

2.5 | Design

Ten groups of seven rats (three males and four females) unknown to each other before the experiment were run in the semi-natural

TABLE 2 Ethogram, definition of recorded behaviors

Category	Behavior pattern	Definition
Female sexual behaviors	*Lordosis; f*	*Posture of the female arching her back, exposing her vagina.*
	Paracopulatory behaviors; f,d	*Approach to a male followed by runaway, often associated with hops, darts, and ear wiggling*
	Rejection; f	*Female kicks, boxes or assumes a belly up posture.*
Male sexual behaviors	*Mounts; f*	*Male stands on its hind legs and places its forepaws on another rat's rump from behind and displays pelvic thrusting*
	Anogenital sniffing; f,d	*Male sniffs the anogenital zone of a female by putting his snout under her tail*
	Pursuit; f,d	*Male runs after a female with his snout close to the anogenital zone of the female*
Prosocial behaviors	Resting with another rat; f,d	Rat rests, laying at a distance shorter than one body length to other rats
	Sniffing other females; f,d	Snout close to a female, sniffing the fur
	Sniffing males; f,d	Snout close to a male, sniffing the fur
Antisocial behaviors	Nose-off male; f,d	Rat faces a male, nose to nose, heads up, with or without boxing
	Nose-off female; f,d	Rat faces a female, nose to nose, heads up, with or without boxing
	Chase female; f,d	*Rat runs after a female trying to overtake it*
	Chase male; f,d	*Rat runs after a male trying to overtake it*
	Flee from male; f,[a]	Escapes from agonistic interaction by running away or simply turning head away from a male
	Flee from another female; f,[a]	Escapes from agonistic interaction by running away or simply turning head away from a female
Exploratory behaviors and behavioral activity distribution	Sniffing the floor; f,d	Sniffs the floor material with all four paws on the floor.
	Rearing; f,d	Sniffs the air while standing on the hind legs
	Transitions; f	Displays a behavior in a zone different from the one in which the previous behavior was displayed
	Time spent in a zone; d	Sum of the duration of all behaviors performed in each zone of the seminatural environment (see Fig. 1B)
	Walk; f,d	Symmetric forward locomotion, all four paws are moving and the rat remains in constant contact with the floor
	Run; f,d	Asymmetric forward locomotion in faster tempo than walk, all four paws are moving but the rat's pace includes a moment of suspension when all four paws are off the ground
Non-social and maintenance behaviors	Resting alone; f,d	Rat rests, laying at a distance longer than one body length to another rat
	Drinking; f,d	*Self-explanatory*
	Eating food; f.d	*Self-explanatory*
	Self-grooming and scratching; f,d	Self explanatory
Fear- and anxiety-related behaviors	Hide alone; f,d,[a]	Rat lays still with head down and legs under its body in a corner or a nest box, at a distance longer than one body length to another rat
	Huddling; f,d,[a]	Rat lays still with head down and legs under its body in a corner or a nest box, at a distance shorter than one body length to another hiding rat. Several rats can hide together in a stack
	Freezing; f,d,[a]	*Rigid, tense, motionless posture without any movement including those of vibrissa*
	Startle; o,[a]	*Sudden reflex contractions of the major muscles of the body, leading to a little jump on the spot. Only observed in response to onset of the white noise*
	Flight from noise; o,l,[a]	Rat rushes into the burrows at the onset of the white noise. The latency is the time from noise onset until the rat escapes from the open field into the burrow
	*Alertness posture; f,d	Rat stands with head raised and body held still and observes the surroundings. Includes aborted entries in the open area from the burrows

Note: This behavior is also described as 'risk assessment' in our previous studies.

Abbreviations: f, frequency; d, duration; l, latency; o, occurrence.

[a]Behavior appears only after the onset of white noise. Behaviors in italics were rarely observed, thus not included in the statistical analysis.

environment. The video recording started when the animals were introduced at 1.00 PM on day 0. Recording was then continuous for a period of 8 days, when the experiment was terminated. The rats were left undisturbed for 5 days. On day 5, females were injected with either EB, PPT, DPN or peanut oil at 9.00 AM. On day 6, the treatment was repeated at the same time, with the exception of the females having received EB the previous day who got administered oil. On day 7, all females received P at 9.00 AM (Table 1). The males remained untreated. The noise started on day 7 at 4.55 PM and stopped 15 minutes later. The behaviours analysed here were recorded during the minute preceding white noise onset and the first minute following it.

2.6 | Behavioural observations

We used the OBSERVER XT, version 12.5 (Noldus, Wageningen, The Netherlands) for behavioural scoring by an observer blind to treatment. We used a refined ethogram based on that used in a previous study, improved with detailed exploratory and fear-related behaviours (Table 2). The frequency and duration of each behaviour pattern was recorded. This made it possible to calculate the mean duration of each behavioural episode. For each behaviour, we specified the individual initiating it, the individual to whom the behaviour was directed when relevant, and the zone of the semi-natural environment in which the behaviour was performed.

2.7 | Data preparation and statistical analysis

For the evaluation of the effects of white noise and for the sex comparison, the four experimental female groups were collapsed into one female group, which we compared with the male group. When data satisfied criteria for parametric analysis according to Shapiro-Wilk's test, we used two-way ANOVA for repeated measures on one factor. The between factor was sex (male or female) and the within factor was noise exposure (before or during). Post-hoc tests were not necessary because both factors had only two modalities. When data did not satisfy criteria for parametric analysis, noise effect was analysed with Wilcoxon tests, with both sexes collapsed. When a noise effect was detected, we proceed to analyse the effect of noise for each sex separately with Wilcoxon tests. The obtained *P* values were multiplied by the number of comparisons (ie, the Bonferroni correction), before applying the significance criterion ($P < .05$).

The effect of the sex of the individual initiating the behaviour was analysed by Mann-Whitney tests within each period (before and during noise). The resulting *P* values were adjusted with Bonferroni correction. In addition, when relevant, the effect of the sex of the individual to which the behaviour was directed was analysed by Wilcoxon tests, with *P* values adjusted with the Bonferroni correction for four comparisons (2 sexes × 2 periods). Probability to flee from the noise at its onset was analysed with binomial tests.

In addition, we analysed how sex and noise affected the localisation of behavioural activity in the semi-natural environment. First, we grouped the observed behaviours in six categories according to our ethogram (Table 2): anxiety-related, exploratory, non-social, pro-social, anti-social and sexual behaviours. The sum of the duration of the behaviours included in each category was determined for each of the seven zones in the semi-natural environment (Figure 1B). The six categories were then used as dependent variables in one-factor non-parametric multivariate analyses of variance (nparMANOVA).[56] The factor was sex. Each zone was analysed separately, before and during noise. In all these tests, an *F* approximation was used to determine significance. In case of significant omnibus test, the Mann-Whitney test, with *P* values adjusted with the Bonferroni correction for six comparisons (six behavioural categories), was used for evaluating sex differences within each behavioural category.

In the last part of the results, we explored the effect of female treatment on immediate fear reactions. When possible, we used a one-way ANOVA for repeated measures. Separate one-way ANOVAs were used for analysing behaviour occurring before and during exposure to white noise. After significant main effect of treatment, Tukey's honestly significant difference test was used for post-hoc comparisons. When data deviated from normal distribution, we used the non-parametric Kruskal-Wallis test, followed by the Conover post-hoc test in case of significance.

Similarly to that performed with regard to the effect of sex, we analysed how female treatment affected the localisation of behavioural activity in the semi-natural environment using the same non-parametric one-way multivariate test, with treatment (Oil, EB, PPT and DPN) as factor. In case of significant omnibus test, the Kruskal-Wallis test was used to analyse treatment effects within each behavioural category.

Statistical analyses were performed with SPSS, version 26 (IBM Corp., Armonk, NY, USA) and R, version 3.6.2 (R Foundation for Statisitical Computing, Vienna, Austria), as well as core, npmv, and PMCMRplus packages. All reported *P* values are already adjusted, when relevant. This is indicated by (Bonferroni correction$_x$, $P_u = .050$), where *x* is the number of multiple comparisons accounted for and P_u is the uncorrected *P* value.

2.8 | Co-occurrence analysis

Chronological scoring of behavioural activity allowed for the visualisation of clusters of temporally associated behaviours, and therefore how experimental manipulations might have altered the structure of behaviour. This was achieved via an analysis of co-occurrence. This method has been described earlier.[26,28] We used a moving window of four behaviour patterns and determined how often one behaviour pattern occurred together with another in the same window. This is defined as a co-occurrence. The window moved, by steps of one behaviour pattern, over the entire individual record. Treatment or sex and noise condition (before or during) were also included in the matrix. Descending

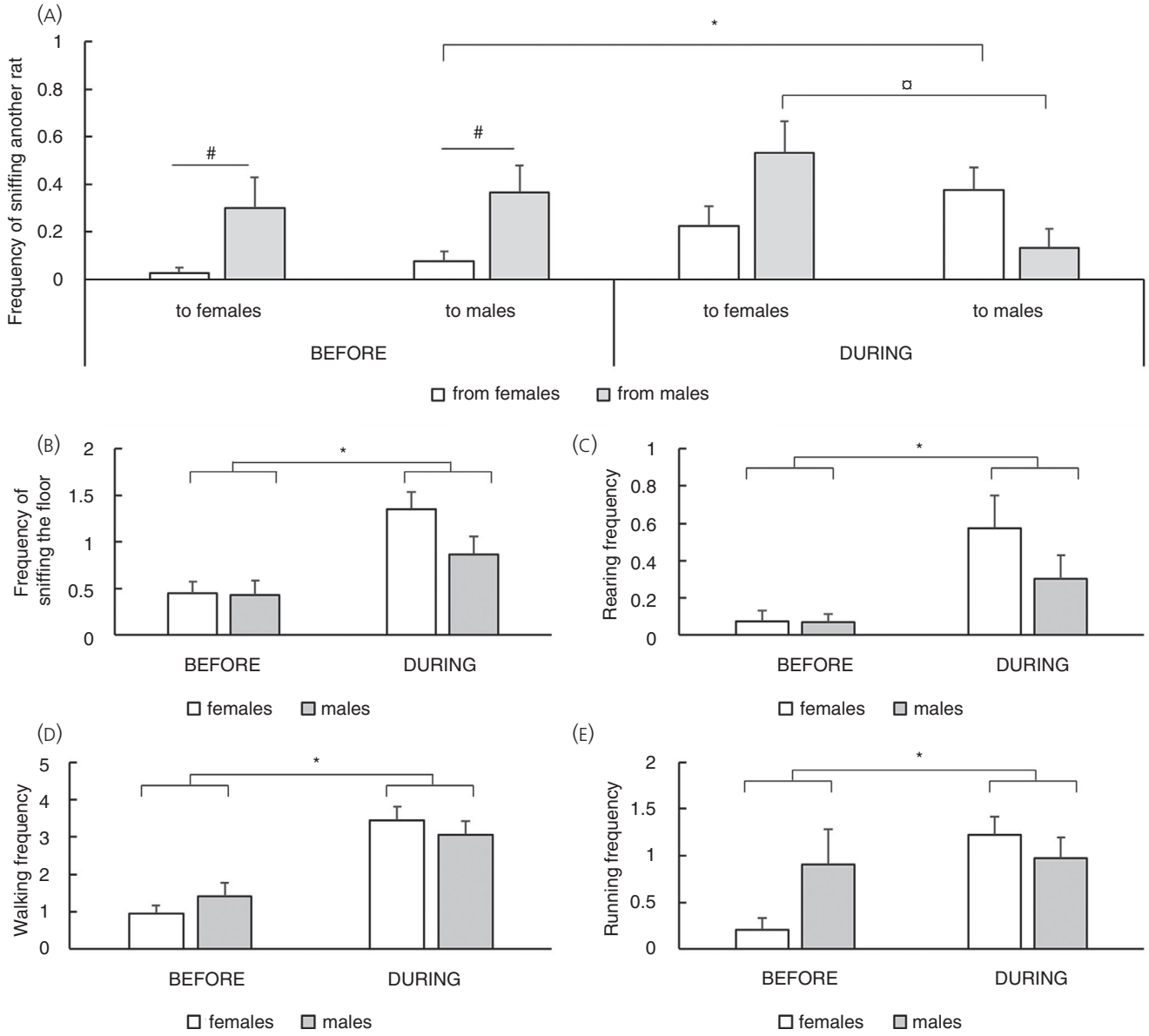

FIGURE 2 Male and female behaviour before and during exposure to white noise. A, Frequency of male and female rats sniffing a conspecific. B, Sniffing the floor. C, Rearing. D, Walking. E, Running. #Effect of initiating individual's sex, $P < .005$. *Effect of noise, $P < .05$. ¤Effect of social partner's sex, $P < .05$. Data are the mean ± SEM. Females, n = 40; males n = 30

hierarchical classification was used to identify clusters of related behaviour.[57,58] The descending hierarchical classification is based on the probability for an item to be proportionally more present in a cluster than it is in the entire data set, as evaluated by chi-squared analysis. Each item is permutated from one cluster to the other to test the robustness of the classification, until statistically independent profiles of items appear.[59] Clusters can therefore be interpreted as groups of individuals and behaviours significantly more co-occurring together than with items of another cluster, as visualised using the Fruchterman-Reingold algorithm.[60]

The criterion for including elements in their respective cluster is a higher frequency of co-occurrence compared to the average occurrence, as well as an association with the cluster determined by chi-squared values equal to or higher than 3.84. This gives an error margin of 0.05 when $df = 1$.[61] Calculations were performed using IRAMUTEQ (Interface de R pour les Analyses Multidimensionnelles de Textes et de Questionnaires; available at http://www.iramuteq.org).

3 | RESULTS

3.1 | White noise immediate effect on male and female behaviour

Here, we only present statistical data concerning the effects of noise. Comparison between sexes and interactions between sex and noise are reported subsequently.

3.1.1 | Pro- and anti-social behaviours

Resting with other rats only occurred before white noise onset (data not shown). Exposure to white noise had no effect on the frequency of *any rat sniffing male conspecifics* ($Z = 0.783$, $P = .434$). Looking at the effect of noise in each sex, exposure to white noise increased the frequency of *female sniffing male conspecific* ($Z = 2.546$, $P = .022$, Bonferroni correction$_2$, $P_u = .011$), although noise had no effect on *males sniffing other males* ($Z = 1.485$, $P = .276$, Bonferroni correction$_2$, $P_u = .138$) (Figure 2A). Exposure to white noise increased the frequency of *any rat sniffing a female conspecific* ($Z = 2.139$, $P = .032$). This did not appear when looking at each sex separately: exposure to white noise had no effect on the frequency of *female sniffing female conspecifics* ($Z = 2.126$, $P = .066$, Bonferroni correction$_2$, $P_u = .033$), nor on *males sniffing females* ($Z = 1.201$, $P = .460$, Bonferroni correction$_2$, $P_u = .230$) (Figure 2A).

Nose-off frequency was similar before and during exposure to white noise, both when directed to females ($Z = 1.698$, $P = .090$) and when directed to males ($Z = 1.293$, $P = .196$). Thus, we did not analyse the effect of noise on each sex separately. *Fleeing from a conspecific* was only observed during exposure to white noise (data not shown).

3.1.2 | Exploratory behaviours, locomotion and spatial distribution of activity

Exposure to white noise increased the frequency of *sniffing the floor* ($F_{1,68} = 20.940$, $P < .001$) (Figure 2B); this was also the case for *rearing* ($Z = 3.100$, $P = .002$) (Figure 2C), *walking* ($F_{1,68} = 55.195$, $P < .001$) (Figure 2D) and *running* ($Z = 3.199$, $P = .001$) (Figure 2E).

The number of *zone transitions* displayed in the semi-natural environment increased during exposure to white noise ($F_{1,68} = 9.258$, $P = .003$) (data not shown). In particular, transitions in the *burrows* increased during white noise exposure ($F_{1,68} = 31.835$, $P < .001$), whereas transitions decreased in the open area ($Z = 4.082$, $P < .001$) (Figure 3A). Regarding spatial distribution of activity, the rats spent more time in the *upper burrows* during exposure to white noise ($F_{1,68} = 19.282$, $P < .001$). By contrast, they spent less time in the *lower burrows* ($F_{1,68} = 6.690$, $P = .012$), the *upper open area* ($Z = 3.833$, $P < .001$), the *lower open area* ($Z = 3.921$, $P < .001$) and the *open area's shelters* ($Z = 3.911$, $P < .001$) (Figure 3B). There was no effect of noise on the time spent in the *doorways* ($Z = 0.255$, $P = .799$) and the *nest boxes* ($Z = 0.078$, $P = .938$).

3.1.3 | Fear-related and non-social behaviours

The frequency of *alertness posture* was higher during white noise exposure ($F_{1,68} = 43.614$, $P < .001$) (Figure 4A). The behaviours *hiding alone* and *huddling* only appeared during white noise. By contrast, resting alone only occurred prior to white noise (Figure 4B). Finally, rats showed more self-grooming before white noise onset than after ($Z = 2.496$, $P = .013$) (Figure 4C).

3.2 | Sex difference in immediate reaction to white noise

3.2.1 | Prosocial behaviours

There was no sex difference in the frequency of resting with another rat before white noise onset ($t_{68} = 0.355$, $P = .724$) (data not shown). Female rats *sniffed female conspecifics* less than males did before white noise onset ($U = 494$, $P = .032$, Bonferroni correction$_2$, $P_u = .016$) but not during exposure to white noise ($U = 463$, $P = .074$, Bonferroni correction$_2$, $P_u = .037$) (Figure 2A). Similarly, females *sniffed male conspecifics* less than males did before ($U = 462$, $P = .024$, Bonferroni correction$_2$, $P_u = .012$) but not during white noise ($U = 481.5$, $P = .098$, Bonferroni correction$_2$, $P_u = .049$) (Figure 2A).

We also analysed the effect of the sex of the individual being sniffed. During white noise, female rats were sniffed by males more often than males were ($Z = 2.637$, $P = .032$, Bonferroni correction$_4$, $P_u = .008$) (Figure 2A). This was not the case before white noise, when females were sniffed equivalently often than males by male rats ($Z = 0.801$. $P = 1$, Bonferroni correction$_4$, $P_u = .423$). Neither before, nor during white noise exposure did we find any effect of the sex of the animal being sniffed by female rats (before: $Z = 1.020$, $P = 1$; Bonferroni correction$_4$, $P_u = .308$; during: $Z = 1.277$, $P = .808$, Bonferroni correction$_4$, $P_u = .202$).

3.2.2 | Antisocial behaviours

There was no sex difference with regard to the frequency of *nose-off directed to females*, neither before ($U = 582$, $P = 1$, Bonferroni correction$_2$, $P_u = .660$, nor during exposure to white noise ($U = 528$, $P = .384$, Bonferroni correction$_2$, $P_u = .192$). Neither did we find any sex difference in the frequency of *nose-off directed to male conspecifics* (before: $U = 585$, $P = .772$, Bonferroni correction$_2$, $P_u = .386$; during: $U = 528$, $P = .202$, Bonferroni correction$_2$, $P_u = .101$) (data not shown). *Fleeing from a conspecific* only occurred during white noise, when males and females fled from female conspecifics equally often ($U = 570$, $P = .539$), and so did they from male conspecifics ($U = 570$, $P = .560$) (data not shown).

3.2.3 | Exploratory behaviours, locomotion and activity spatial distribution

There was no sex effect on the frequency of *sniffing the floor* ($F_{1,68} = 1.687$, $P = .198$) and no interaction between sex and noise exposure ($F_{1,68} = 1.867$, $P = .114$) (Figure 2B). Similarly, there was no effect of sex on *rearing*, neither before ($U = 591$, $P = 1$, Bonferroni

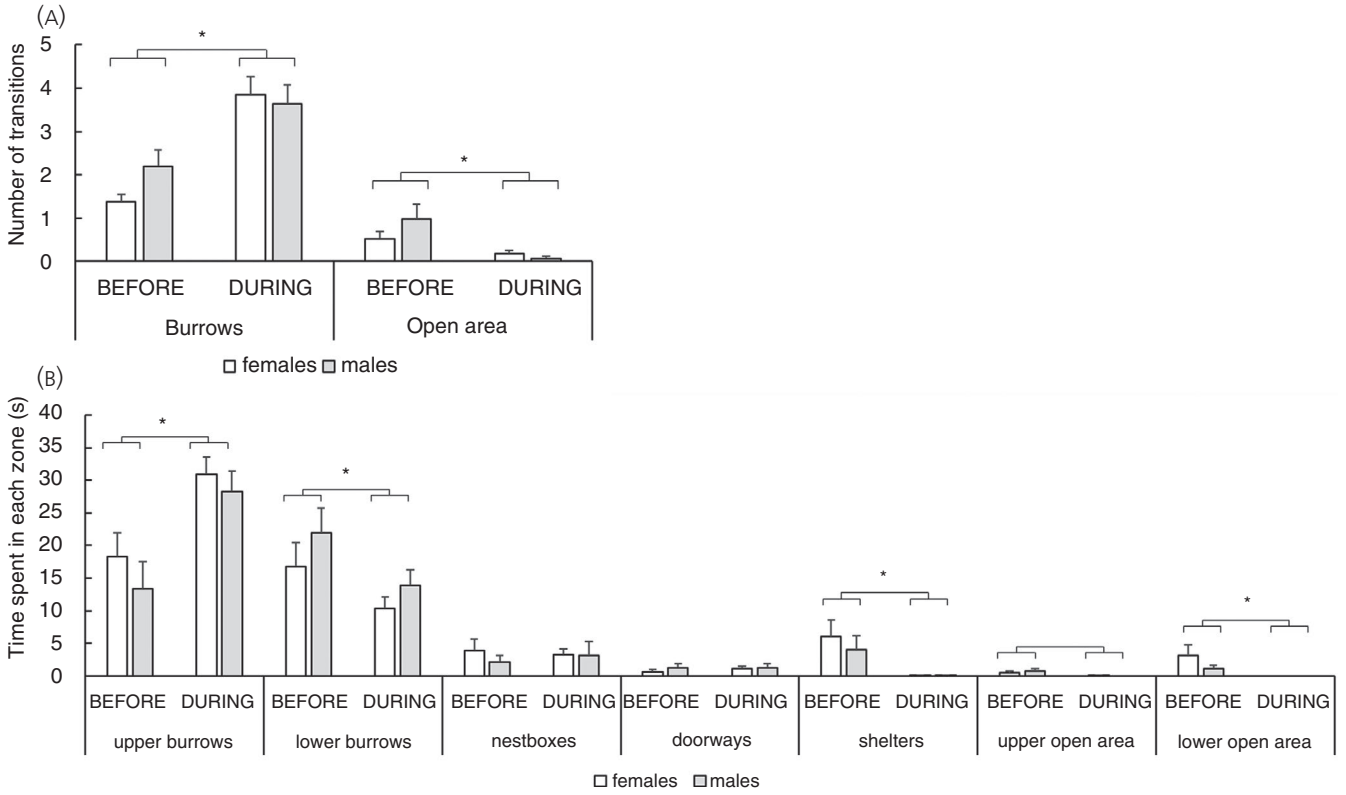

FIGURE 3 Ambulatory activity and spatial distribution of activity, before and during exposure to white noise. A, Number of transitions displayed in the burrows and the open area. B, Time spent in each zone of the semi-natural environment, see Figure 1B for the localisation of the zones and areas mentioned. *Effect of noise, $P < .05$. Data are the mean ± SEM. Females, n = 40; males n = 30

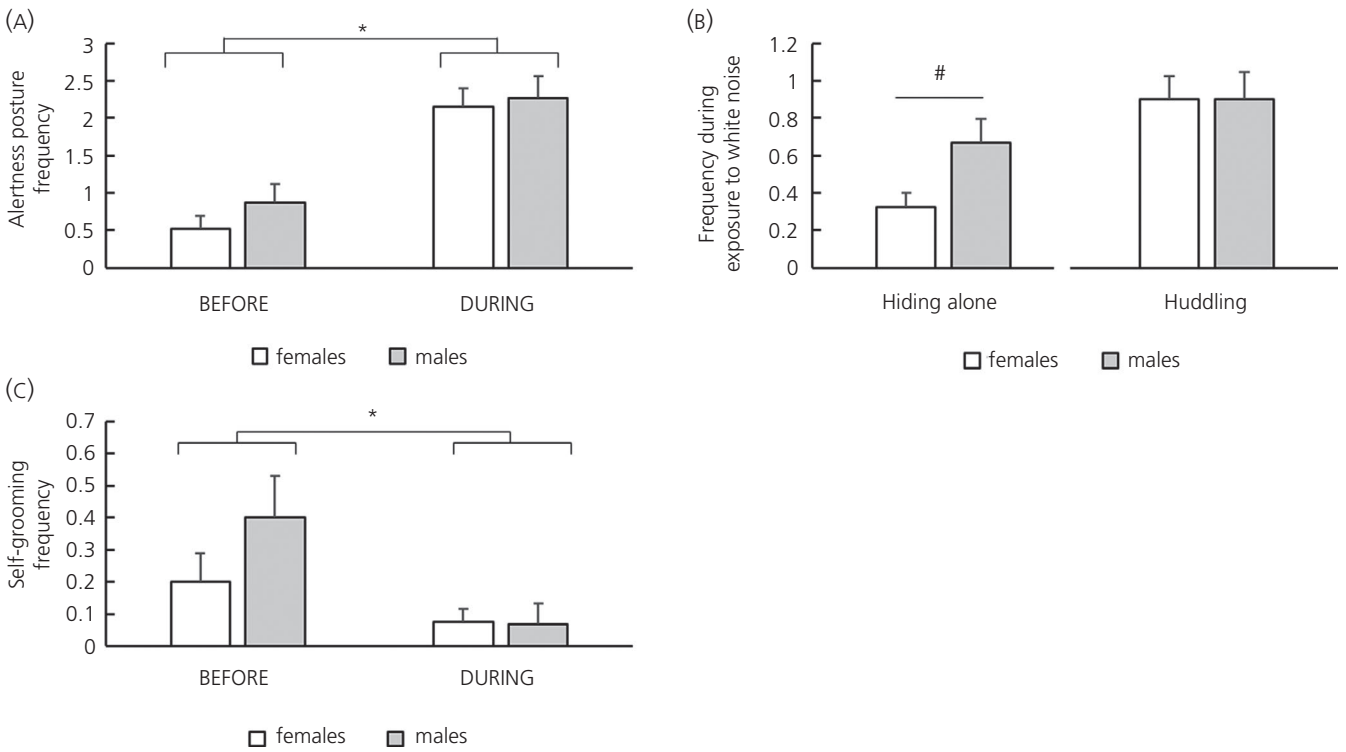

FIGURE 4 Frequency of fear-related and non-social behaviours, before and during exposure to white noise. A, Alertness posture. B, Hiding alone and huddling. C, Self-grooming. #Effect of sex, $P < .05$. *Effect of noise, $P < .05$. Data are the mean ± SEM. Females, n = 40; males n = 30

correction$_2$, P_u = .791), nor during white noise (U = 533.5, P = .606, Bonferroni correction$_2$, P_u = .303) (Figure 2C).

Sex did not influence the frequency of *walking* ($F_{1,68}$ = 0.007, P = .931) and we found no interaction between sex and noise exposure for this behaviour ($F_{1,68}$ = 2.208, P = .142) (Figure 2D). Similarly, we found no effect of sex on *running*, neither before (U = 515, P = .222, Bonferroni correction$_2$, P_u = .111), nor during white noise (U = 514, P = .562, Bonferroni correction$_2$, P_u = .281) (Figure 2E).

Sex did not modify the number of *transitions* displayed in the semi-natural environment ($F_{1,68}$ = 1.013, P = .318) and there was no interaction between sex and noise exposure for transition frequency ($F_{1,68}$ = 3.319, P = .073) (data not shown). There was no sex effect on the transition frequency in the *burrows* ($F_{1,68}$ = 0.656, P = .421), nor any interaction between sex and noise exposure ($F_{1,68}$ = 2.261, P = .137). Transitions in the *open area* were unaffected by sex (before: U = 559.5, P = 1, Bonferroni correction$_2$, P_u = .539; during: U = 577, P = 1, Bonferroni correction$_2$, during: P_u = .574) (Figure 3A).

There was no effect of sex on the *time spent in the different zones* of the semi-natural environment (all P > .056) and no interaction between sex and noise on the time spent in the upper burrows ($F_{1,68}$ = 0.139, P = .711) and in the lower burrows ($F_{1,68}$ = 0.078, P = .781) (Figure 3B).

3.2.4 | Fear-related behaviours

Male and female *probability to flee* from the noise at its onset did not differ (Binomial test, P = .468). The *latency to flee* from the noise was no different between males and females (Z = 0.688, P = .491) (data not shown). There was no main effect of sex on the frequency of *alertness posture* ($F_{1,68}$ = 0.784, P = .379), and no interaction between sex and noise ($F_{1,68}$ = 0.241, P = .625) (Figure 4A). *Hiding alone* was displayed by males more often than by females (Z = 2.064, P = .039) (Figure 4B). Sex had no effect on *huddling* frequency (t_{68} = 0, P = 1) (Figure 4B).

3.2.5 | Non-social behaviours

Resting alone showed no sex effect (t_{68} = 1.712, P = .091), and males and females *self-groomed* equally often (before: U = 1.669, P = .190, Bonferroni correction$_2$, P_u = .094; during: U = 0.693, P = .976, Bonferroni correction$_2$, P_u = .488) (Figure 4C).

3.2.6 | Localisation of male and female behavioural activity

Before exposure to white noise, we found an effect of sex on the time spent displaying the six different behavioural categories in the *lower burrows* ($F_{3.37, 224.16}$ = 2.291, P = .019). Univariate analyses revealed that males displayed more prosocial behaviours than females in this zone (U = 810.5, P = .019, Bonferroni correction$_6$, P_u = .003). This was associated with a 67.54% probability for this behavioural category to be displayed by a male in this area compared to randomly selected behavioural categories by either sex, according to the relative effects reported by the nparMANOVA. No sex difference appeared in the other behavioural categories (all P = 1, Bonferroni correction$_6$, all P_u > .225). The nparMANOVA comparing sex differences among behavioural categories was non-significant in all other zones before exposure to white noise (all P > .271) (Figure 5A).

Finally, during exposure to white noise, multivariate analysis reported a sex effect in the nest boxes ($F_{1.81, 120.17}$ = 4.715, P = .013) but not in the other zones (all P > .080). However, univariate analyses failed to detect any significant sex effect on the behavioural categories displayed in the nest boxes (all P > .136, Bonferroni correction$_6$, P_u > .023), even though relative effects reported that exploratory behaviours occurring in the nest boxes had a 62.08% probability of being displayed by a female compared to randomly selected behavioural categories by either sex (Figure 5B).

3.2.7 | Co-occurrence analysis

Male and female rats appeared in two different clusters before white noise onset. Males were associated with most exploratory and ambulatory behaviours, with prosocial behaviours as well as with self-grooming. Female rats were associated with all anti-social behaviours, resting behaviours, and with the alertness posture (Figure 6A).

During exposure to white noise, male rats appeared in a distinct cluster only including sniffing female conspecifics and nose-off to other males. The cluster of behaviours associated with female rats showed a more extensive behavioural repertoire, including other pro- and anti-social behaviours, and all exploratory, ambulatory and fear-related behaviours (Figure 6B).

3.3 | Treatment effect on female immediate reaction to noise

3.3.1 | Pro- and antisocial behaviours

During exposure to white noise, the frequency of *sniffing a male* conspecific differed between the treatments (χ^2 = 8.101, df = 3, P = .044). Females treated with PPT sniffed males more frequently than those treated with oil (P = .016) and EB (P = .011) (Figure 7A). We did not observe any other difference between treatment groups in social behaviours before or during white noise (all P > .392). Additionally, female treatment did not affect the frequency of being sniffed by other rats (all P > .505), nor that of receiving nose-off (all P > .533) (data not shown).

3.3.2 | Exploratory behaviours, locomotion and spatial distribution of activity

There was no difference between the treatments in the *total number of transitions* between zones of the semi-natural environment

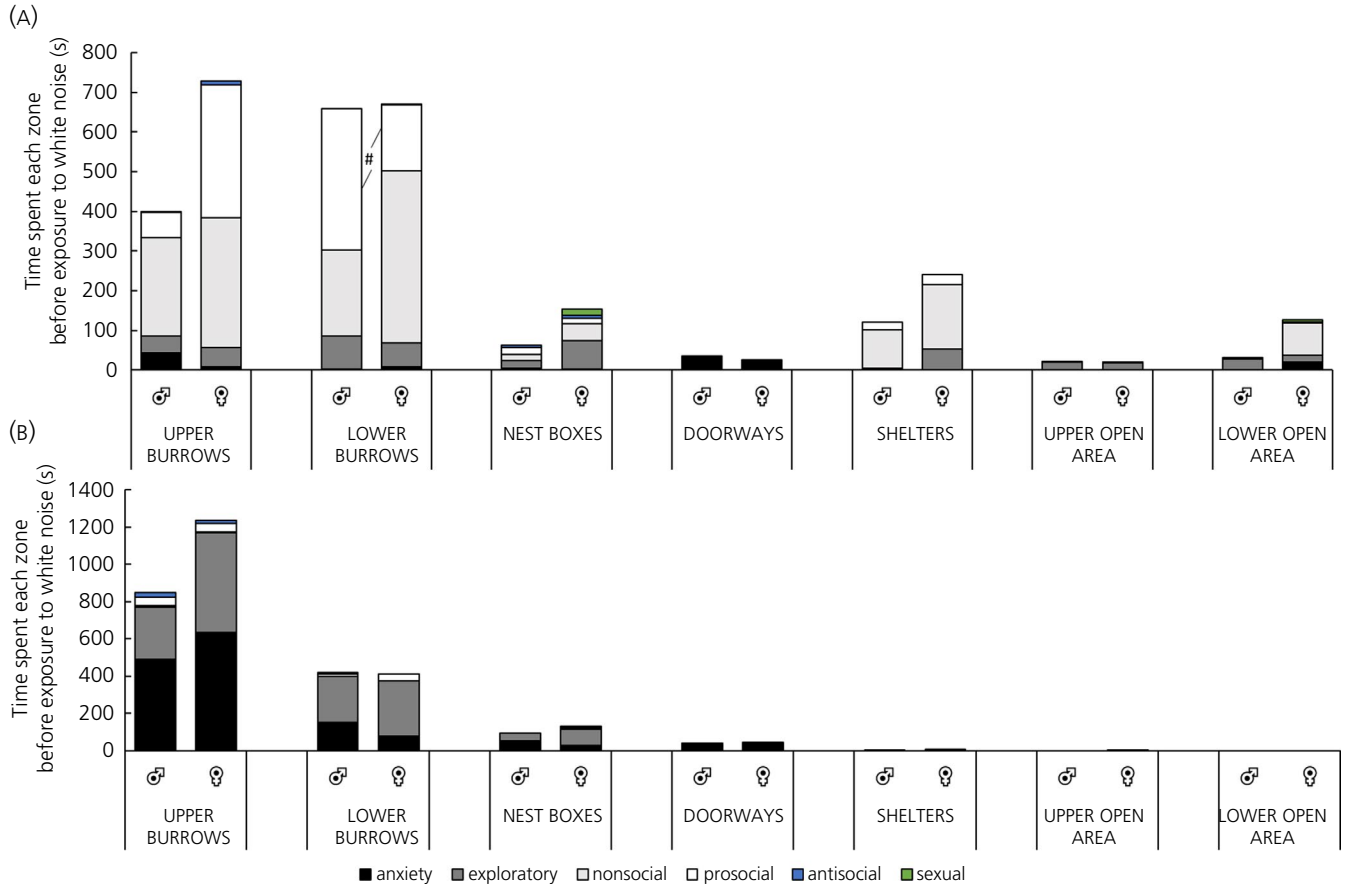

FIGURE 5 Localisation of behaviours displayed by males and females in each of the seven zones of the semi-natural environment. A, Cumulated time spent displaying each category of behaviour before exposure to white noise. B, Cumulated time spent displaying each category of behaviour during exposure to white noise. #Effect of sex, $P < .05$. Females, n = 40; males n = 30

($F_{3,36}$ = 1.038, P = .387). However, looking at *transitions within the open area*, we found a treatment effect before white noise onset (χ^2 = 9.418, df = 3, P = .024). Before the beginning of white noise, females treated with EB displayed more transitions in the open area than females treated with oil (P = .006) and DPN (P = .019). Females treated with PPT also displayed more transitions than those treated with oil (P = .038) (Figure 7B). Furthermore, there was a treatment effect on the time spent in the *lower open area* before white noise onset (χ^2 = 10.789, df = 3, P = .013). The EB group spent more time in that area than the oil (P = .003) and the DPN groups (P = .003) (Figure 7C). No other difference between treatment groups was found in exploratory behaviours and activity spatial distribution (all $P > .076$).

3.3.3 | Fear-related behaviours

Only PPT-treated females showed a high probability to flee from the noise at its onset (Binomial test, P = .019); other treatment groups did not differ from the mean flight probability (all $P > .227$). In addition, the latency to flee from the noise was different between the groups (X^2 = 9.064, df = 3, P = .028). PPT-treated females had a shorter latency to flee from the noise than Oil- (P = .004), EB- (P = .021) and DPN-treated females (P = .023). Other groups did not differ from each other (all $P > .490$) (Figure 7D). We found no other treatment effect on fear-related behaviours (all $P > .392$).

3.3.4 | Other behaviours

There was no difference between the treatment groups for *antisocial behaviours* (all $P > 0.076$) and *non-social behaviours* (all $P > .426$) (data not shown).

3.3.5 | Localisation of female behavioural activity

The nparMANOVA used to compare treatment effects within each of the seven zones of the semi-natural environment before exposure to white noise reported significant differences between treatments in the lower burrows ($F_{10.89, 130.65}$ = 2.291, P = .014). However, univariate tests did not show any significant effect of treatment within any behavioural category (all $P > .141$, Bonferroni correction$_6$, $P_u > .024$). The nparMANOVA was not significant for any other zone (all $P > .058$) (Figure 8A). During exposure to white noise, multivariate analysis did not find any significant treatment effect in any of the zones of the semi-natural environment (all $P > .266$) (Figure 8B).

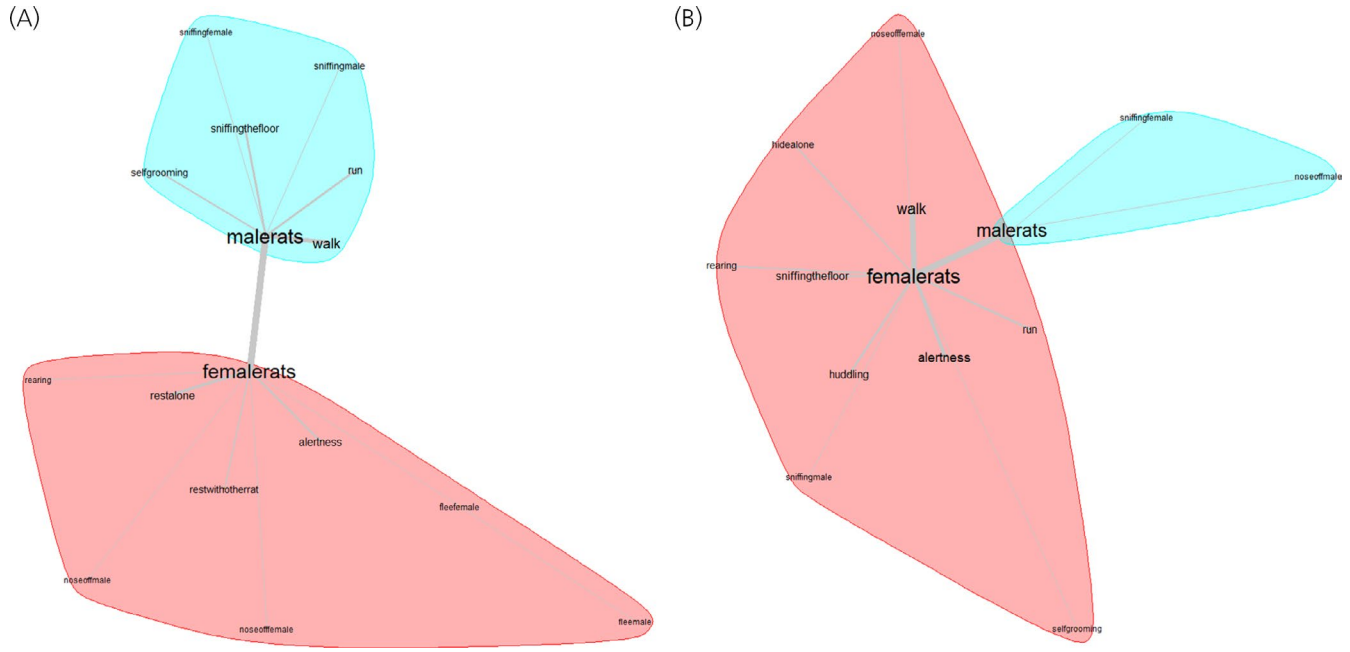

FIGURE 6 Co-occurrence analysis showing main behavioural associations typical of each sex. Clusters of behavioural association are represented in halos of different colours. The size of the words is proportional to their occurrence frequency. The thickness of the branches is proportional to the frequency of association of the two items linked. A, Before white noise onset. B, During exposure to white noise. Females, n = 40; males n = 30

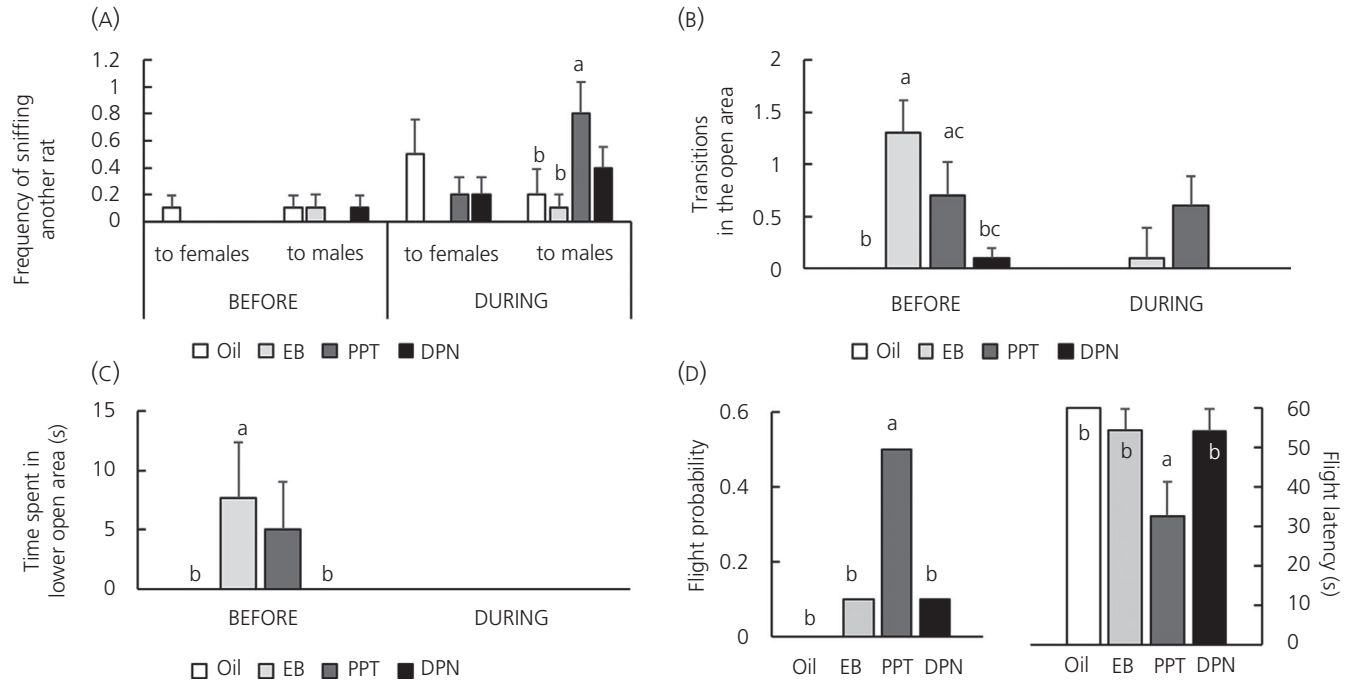

FIGURE 7 Treatment effect on female behaviour, before and during exposure to white noise. A, Female frequency of sniffing another rat. B, Transitions in the open area. C, Time spent (s) in the lower open area. D, Probability and latency (s) to flee from the noise at its onset. Different letters indicate a significant difference, $P < .05$. Data are the mean ± SEM. DPN, diarylpropionitrile, n = 10; EB, 17β-oestradiol benzoate, n = 10; Oil, peanut oil, n = 10; PPT, propylpyrazoletriol, n = 10

3.3.6 | Co-occurrence analysis

Before white noise onset, females treated with Oil, PPT and DPN appeared in the same cluster associated with most pro- and anti-social behaviours, as well as with resting alone. Females treated with EB formed a separate cluster including all exploratory and fear-related behaviours, nose-off to males and self-grooming (Figure 9A).

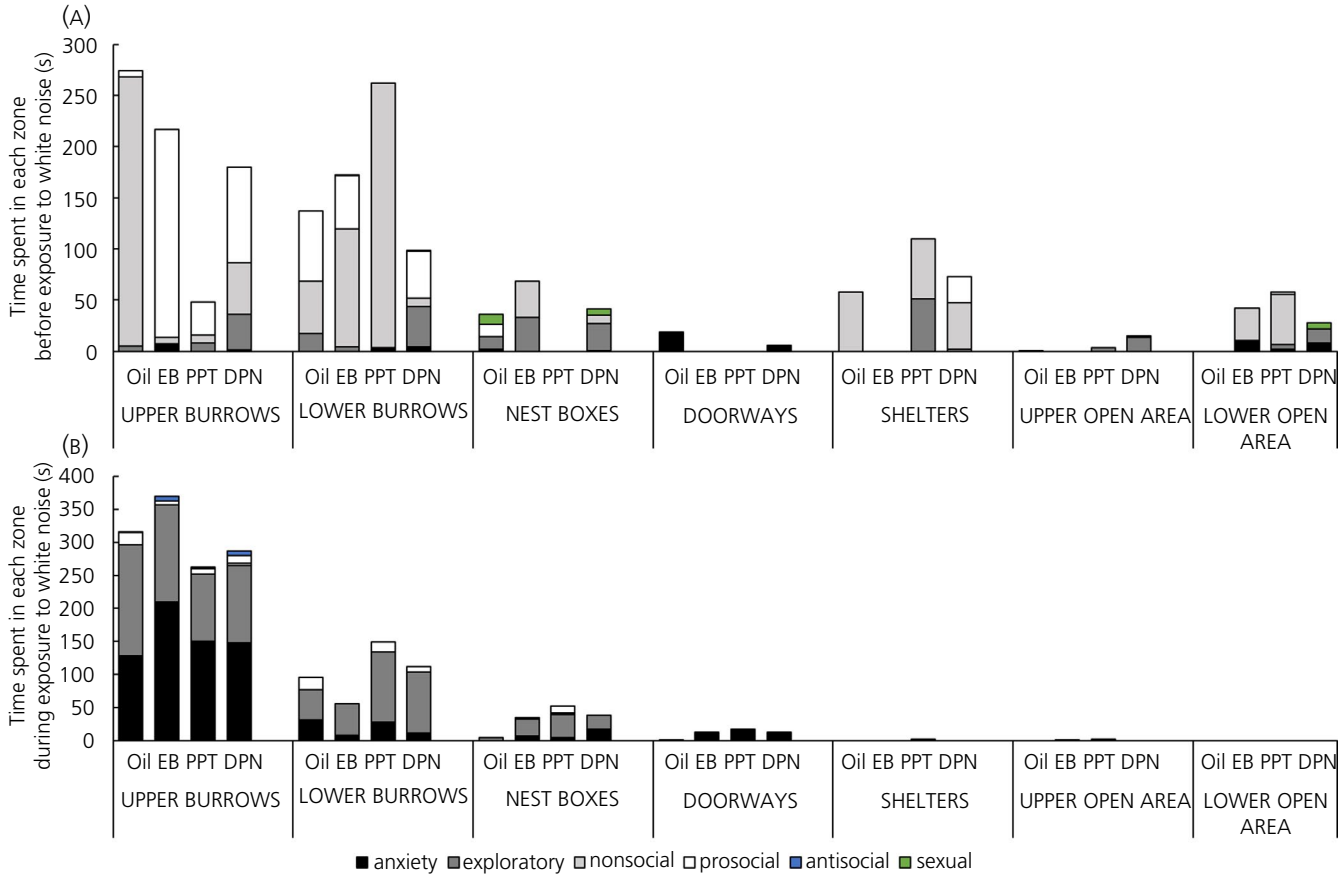

FIGURE 8 Localisation of behaviours displayed by female experimental groups in each of the seven zones of the semi-natural environment. A, Cumulated time spent displaying each category of behaviour before exposure to white noise. B, Cumulated time spent displaying each category of behaviour during exposure to white noise. DPN, diarylpropionitrile, n = 10; EB, 17β-oestradiol benzoate, n = 10; Oil, peanut oil, n = 10; PPT, propylpyrazoletriol, n = 10

During exposure to white noise, females treated with EB remained in a separate cluster including the exploratory behaviour 'sniffing the floor', and nose-off to males. The PPT group also formed a distinct cluster, associated with fear-related and exploratory behaviours. Finally, the Oil and DPN groups belonged to the same cluster with the fear-related behaviour 'hiding alone' and prosocial behaviours directed to males and females (Figure 9B).

4 | DISCUSSION

The effects of white noise and the sex comparisons are summarised in Table 3.

4.1 | Immediate reaction to white noise

Exposure to white noise produced clear immediate reactions. The rats fled from the open area into the burrow system, and there they preferred to spend their time in the zone farthest from the entrances to the open area. Even the shelters available in the open area were abandoned. The noise-induced avoidance of the open area did not suppress exploratory activity in the burrow. By contrast, the frequency of sniffing the floor, rearing, walking and running strongly increased during exposure to white noise. Increased exploratory behaviours have been reported earlier during sustained white noise exposure in an open field[62] or in a test box of the size of the regular home cage.[63] In addition to modifying exploration within the burrow, the aversive stimulus elicited the fear-related behaviours hiding alone and huddling and enhanced the frequency of the alertness posture, whereas resting was suppressed.

It is important to observe that the noise intensity was similar in the burrow and the open area. Thus, the rats could not escape from the aversive stimulus by entering the burrow and spend their time far away from the openings. In addition to interpreting the immediate behavioural effects of noise as simple escape responses, they can also be regarded as manifestations of a fear reaction to a sudden aversive stimulus. Open spaces are avoided,[64,65] and both locomotor activity and reactivity to environmental stimuli are enhanced in 'safe' areas because of heightened general arousal.[30,66] It is well established that fear and anxiety are associated with heightened arousal.[38,67] The immediate response to the noise is similar to the sustained response (ie, behaviour observed during a continuous 15-minute exposure) reported in earlier studies.[26,27] This suggests

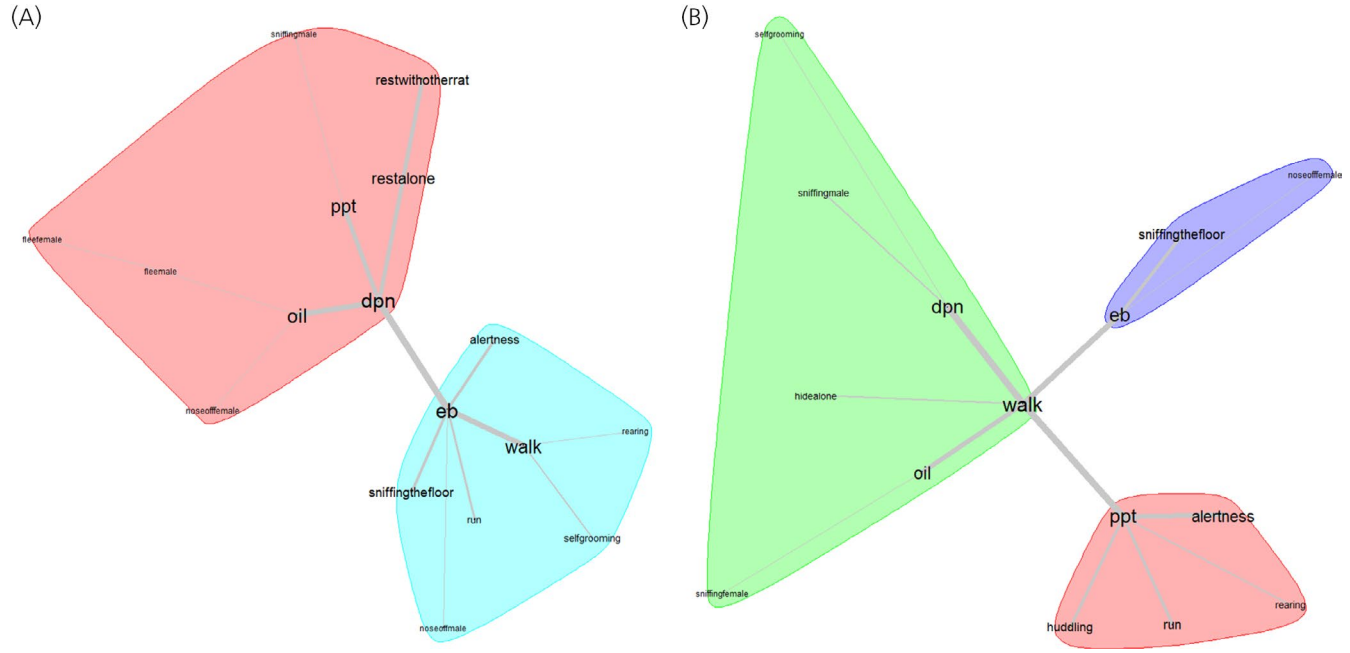

FIGURE 9 Co-occurrence analysis showing main behavioural associations typical of each of the female treatment group. Clusters of behavioural association are represented in halos of different colours. The size of the words is proportional to their occurrence frequency. The thickness of the branches is proportional to the frequency of association of the two items linked. A, Before white noise onset. B, During exposure to white noise. Oil, n = 10; EB, n = 10; PPT, n = 10; DPN, n = 10

that there is no habituation to the aversive stimulus during an exposure of that length. It may also be noted that some behaviours, such as huddling, returned to pre-noise levels within 1 minute after noise offset. Visits to the open area recovered more slowly, with recovery needing more than 5 minutes.[28] The latter observations indicate that the white noise did not cause a lasting fear or anxiety reaction. Indeed, most of the responses shown appear to require the presence of the aversive stimulus.

An important question is whether the data from the semi-natural environment offer any information about the responses to white noise not already obtained in simpler procedures. The most important difference between the present procedure and traditional tests such as the elevated plus maze or the light/dark choice test is that the rats in our procedure have the opportunity to express a substantial proportion of their behavioural repertoire. The large and physically complex environment also allows the rats to make differential use of space, according to circumstances. Finally, the mixed sex groups used here make it possible to observe interactions within the same sex as well as between sexes. The results of the present experiment show that white noise simultaneously affects the use of space, the amount and distribution of ambulatory activity and social interactions. This rather complete picture of the effects of white noise could not have been obtained in any of the traditional procedures. It is also noteworthy that white noise did not produce any freezing response in our procedure. Indeed, freezing was so unusual that it could not even be analysed. In traditional procedures, freezing is a prominent response to white noise.[68] Similarly, fox odour causes freezing in several tests[69,70] but not in the semi-natural environment.[26] We have suggested that phenomena such as social buffering[71] or a sense of controllability,[72,73] present in the semi-natural environment but absent in traditional tests, can explain the low incidence of freezing in the former. Not finding an expected response may be as informative as finding an unexpected response. In this particular case, it shows that a fear or stress response depends on the social or physical context. Because of these and similar observations, we propose that data from the semi-natural environment have larger generalisability to natural contexts than data from other procedures.

4.2 | Sex difference in immediate fear reactions

Both before and during the noise, there were few sex differences (Table 3). One of the few differences was that the males sniffed females more than the females did before the noise. Curiously, they also sniffed the other males more than the females did. It appears that the males were more sociable than the females. This coincides with earlier data from the social interaction test.[74] Males spent more time in social interactions than females did. The larger sociability in males is dependent on testicular hormones because castration reduces social interaction to the female level. Interestingly, ovarian hormones do not modify female sociability because intact and ovariectomised females show the same level of social interaction.[75] Furthermore, ovariectomised females treated with testosterone show the same level of social interaction as intact males.[76] It thus appears that testosterone leads to high sociability both in males and females. It is worthy of note that the sex difference in social investigation observed in the semi-natural environment

TABLE 3 Effect of sex and noise on behavioural expression

Behavioural category	Behaviour pattern examined	Expression before exposure to white noise	Expression during exposure to white noise
Prosocial	Resting with another rat[F]	F = M	ND
	Sniffing a female[F]	**F < M**	F = M
	Sniffing a male[F]	**F < M**	⇑ F = M
Antisocial	Nose-off to a female[F]	F = M	F = M
	Nose-off to a male[F]	F = M	F = M
	Flee from a female[F]	ND	F = M
	Flee from a male[F]	ND	F = M
Exploratory	Sniffing the floor[F]	F = M	⇑ F = M ↑
	Rearing[F]	F = M	⇑ F = M ↑
	Walk[F]	F = M	⇑ F = M ↑
	Run[F]	F = M	⇑ F = M ↑
	Transitions in the semi-natural environment[F]	F = M	⇑ F = M ↑
	Transitions in the burrows[F]	F = M	⇑ F = M ↑
	Transitions in the open area[F]	F = M	⇓ F = M ↓
	Time spent in upper burrows[S]	F = M	⇑ F = M ↑
	Time spent in lower burrows[S]	F = M	⇓ F = M ↓
	Time spent in a nest boxes[S]	F = M	F = M
	Time spent in doorways[S]	F = M	F = M
	Time spent in shelters[S]	F = M	⇓ F = M ↓
	Time spent in upper open area[S]	F = M	⇓ F = M ↓
	Time spent in lower open area[S]	F = M	⇓ F = M ↓
Fear- and anxiety-related	Hide alone[F]	ND	**F < M**
	Huddling[F]	ND	F = M
	Alertness posture[F]	F = M	⇑ F = M ↑
Non-social	Resting alone[F]	F = M	ND
	Self-grooming[F]	F = M	⇓ F = M ↓

Note: Behavioural expression was measured in frequency ([F]) or in duration in seconds ([S]). When possible, the effects of sex and noise were analysed by a two-ways ANOVA for repeated measures on one factor. Otherwise, the effect of sex was analysed by Mann-Whitney tests, and that of noise by Wilcoxon tests. In the case of a significant sex effect, the effect of noise was analysed in each sex separately; otherwise, both sexes were collapsed in the analysis. Sex differences are indicated in bold. Noise effect is indicated by up and down arrows representing increased (up) and decreased (down) display of the examined behaviour, compared with the period preceding noise. Noise effect on female (⇓ ⇑) and male (↑ ↓) behavioural expression. ND = behaviour pattern not displayed. Any behaviours that are not here did not show any noise or sex effect.

before noise exposure is similar to that observed in completely different procedures. During the noise, males preferred sniffing females, whereas females showed no preference for sniffing a particular sex. Similarly, the females hid less alone than the males did. It thus appears that, with these exceptions, males and females behave in a similar way when exposed to white noise in the semi-natural environment.

The similarity between the sexes in behavioural responses to fear coincides with the similarity in the endocrine response. Although no data are available from the semi-natural environment, corticosterone and adrenocorticotrophic hormone are released after white noise of approximately the same intensity as used in the present study in both sexes.[77-79] Unfortunately, males and females were evaluated in different studies, making direct sex comparisons impossible, although it is evident that both sexes show a robust, endocrine stress response when exposed to white noise. In this context, it is important to note that there are sex differences with regard to the regulation of the corticotrophin-releasing factor response to stress, which may lead to increased stress sensitivity in females.80 However, even though such differences are likely, they do not appear to alter the immediate response to white noise. Indeed, the present data, together with earlier studies of sex differences, suggest that such differences are much more prominent with regard to sustained stress than to phasic stress.

The modest influence of sex on the behavioural responses to white noise coincides with other data showing small sex differences in behaviour in classical anxiety tests. A review of the subject revealed many contradictory observations, and concluded that novel

test procedures and novel statistical analyses are required before any conclusion can be reached.[81] Another review also found many inconsistent observations. In some tests, females appeared more reactive than males to anxiety-provoking situations; in others, they were less reactive than males.[82] The lack of consensus concerning possible sex differences appears to persist. One recent study in male and female rats illustrates this.[12] The results showed that females spent more time than males on the open arms of an elevated plus-maze, although there was no sex difference in behaviour in the open field. Similarly, in a variant of the social interaction test, there was no sex difference with regard to approach to a conspecific confined in a cage.[12] This differs from the reliable sex difference found when direct physical interaction is possible (see above). It appears that sex differences often are limited to specific tests. One reason for the persistent confusion may be the use of tests lacking external validity. Only the future will tell us whether the semi-natural environment can offer more consistent data.

4.3 | Effect of ERs on female immediate fear reactions

Several of the neural responses to stress are modulated by oestrogens.83 Even though the role of the different oestrogen receptors is unclear, there are some data showing that they may have opposite effects on neural responses. For example, ERα increases, whereas ERβ reduces, the expression of tyrosine hydroxylase.[84] Oestrogens also affect serotonergic functions[84] and, in that case, ERα and ERβ have different effects.[85] The different or opposing effects on neural mechanisms coincide with the different or opposing effects of the oestrogen receptors on behavioural fear responses. As noted earlier in the Introduction, there are several reports of anxiolytic and anxiogenic effects of selective ER agonists in various tests for anxiety. In the present study, the only effect of the agonists on behaviour during white noise exposure was a higher frequency of sniffing the males in females treated with the ERα agonist PPT than in females treated with oil. This could perhaps be attributed to the fact that the dose of PPT used here also stimulates female sexual behaviour.[27] Unfortunately, this explanation cannot be correct because PPT-treated females were also superior to the EB-treated females, and the EB dose used stimulates sexual behaviour to the same degree as PPT. Consequently, it is difficult to explain the effect of PPT in terms of enhanced sexual behaviour. However, the fact that the females given EB and PPT displayed higher activity in the open area than the other groups before the onset of white noise can be caused by their sexual receptivity. It was reported previously that sexually receptive females are more active in the open area than non-receptive females.[24]

The limited effects of the ER agonists (and of EB itself) on the responses to white noise may appear to contradict the many earlier studies reporting their anxiolytic or anxiogenic effects. However, the effects of ERs on anxiety are complex, with the ERα considered to be anxiogenic in certain contexts, whereas the ERβ is always anxiolytic.[86] Because endogenous oestrogens are acting at both receptors simultaneously, it is extremely difficult to predict the net effect of oestrogen actions. It is possible that the many effects of the administration of selective ER agonists are purely pharmacological. This notion is supported by recent data. A carefully conducted study in male and female rats failed to detect any effect of the oestrus cycle on behaviour in the elevated plus-maze, in the open field test, or in the social interaction test.[12] These data would certainly speak against any functionally significant role of oestrogens for the behaviour displayed in these tests. This conclusion is reinforced by data from a study performed in male and female mice lacking either the ERα or the ERβ. Neither of these mice were different from wild-type in the open field, light/dark choice test or in the elevated plus-maze.[87] The studies outlined above strengthen the notion that it is difficult to formulate founded hypotheses concerning anxiolytic and anxiogenic effect of oestrogens. However, in our previous study[27] of sustained fear or anxiety during noise exposure, we found that PPT enhanced the probability for escape from the noise and reduced the latency to escape. Furthermore, in the co-occurrence analysis, PPT formed a separate cluster associated with fear-related behaviours. This was interpreted as an anxiogenic effect.[27] Also, in the present study, we found that PPT formed a cluster separate from oil and DPN during but not before noise exposure, and that the behaviours in the PPT cluster were mostly related to a fear reaction. It appears that the ERα agonist heightened fear responses already during the first min of noise (present study) and that these responses persisted during the entire exposure (ie, during sustained fear or anxiety).[27]

The entirely negative results obtained in previous studies[12,87] are difficult to explain. They do not coincide with the results of either the present study or those of our previous studies in which we also found an anxiogenic effect of the ERα in fear-inducing contexts.[27,28,88] We propose that the semi-natural environment is more appropriate for detecting subtle effects than the classical anxiety tests.

5 | CONCLUSIONS

One of the essential elements in the present study is that we evaluated behaviour displayed in response to a sudden, aversive stimulus in a familiar environment, in rats living in a mixed sex group. In the most commonly used procedures for studying anxiety, the experimental subject is introduced into a novel, often aversive, situation. Thus, reactions to novelty are superimposed on possible reactions of fear. Furthermore, the experimental subject is tested alone, whereas it is known that rats are gregarious, and that group-living is an integral part of their natural habitat.

The importance of these essential elements is not known, although their presence should assure external validity in the brunswikian sense, whereas their absence should reduce that validity, making generalisations between experimental procedures risky and translational relevance limited.

The immediate responses to an aversive stimulus, or phasic anxiety, are similar in male and female rats. Moreover, these immediate responses are similar to those recorded during a long period of noise exposure. Thus, there is no habituation to the aversive stimulus. It has been speculated that phasic and sustained anxiety have different neurobiological bases. Phasic anxiety should be mediated by the central nucleus of the amygdala, whereas sustained anxiety is assumed to be mediated by the bed nucleus of the stria terminalis.[38] This may well be the case for anxiety produced in other procedures, particularly conditioned anxiety or fear responses,[39] although it does not appear to apply with respect to the response to a strongly aversive stimulus in a familiar environment. It is quite unlikely that different neural systems should provoke highly similar behavioural responses. Indeed, the different functions of the central nucleus of the amygdala and the bed nucleus of the stria terminalis in fear and anxiety reactions has been questioned.[89] The present data do not contradict this proposal.

The anxiogenic action of the ERα was confirmed, whereas the purported anxiolytic action of the ERβ failed to appear. Indeed, we have never been able to find any effect of this receptor in the semi-natural environment. The implications of this failure are unclear. However, considering our use of a procedure with external validity (sometimes called ecological validity), it might be reasonable to question the robustness of the actions of the ERβ related to fear and anxiety.

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CONFLICT OF INTERESTS

The authors declare that they have no conflicts of interest.

PEER REVIEW

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DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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