Research report

Estrogen receptors α and β in the central amygdala and the ventromedial nucleus of the hypothalamus: Sociosexual behaviors, fear and arousal in female rats during emotionally challenging events

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A B S T R A C T

Estrogens receptors (ER) are involved in several sociosexual behaviors and fear responses. In particular, the ERα is important for sexual behaviors, whereas ERβ modulates anxiolytic responses. Using shRNA directed either against the ERα or the ERβ RNAs (or containing luciferase control) encoded within an adeno-associated viral vector, we silenced these receptors in the ventromedial nucleus of the hypothalamus (VMN) and the central amygdala (CeA). We exposed ovariectomized female rats, sequentially treated with estradiol benzoate and progesterone, to five stimuli, previously reported to elicit positive and negative affect. The subjects were housed in groups of 4 females and 3 males in a seminatural environment for several days before hormone treatment. We analyzed the frequency of a large number of behavior patterns. In addition, we performed analyses of co-occurrence in order to detect changes in the structure of behavior after infusion of the vectors. Silencing the ERα in the VMN disrupted lordosis and showed some anxiolytic properties in aversive situations, whereas silencing of the ERβ in this structure had no effect. This was also the case after silencing the ERα in the CeA. Silencing of the ERβ in this structure increased risk assessment, an expression of anxiety, and increased olfactory exploration of the environment. We hypothesize that the ERβ in the CeA has an important role in the well-established anxiolytic effects of estrogens, and that it may modulate arousal level. Furthermore, it seems that the ERα in the VMN is anxiogenic in aversive or threatening situations, in agreement with other studies.

1. Introduction

Estrogen receptors (ERs) play an important role in the modulation of female sexual and social behaviors. The ERα is crucial for sexual behaviors, determining both receptivity and sexual approach behaviors [1–6]. These effects are mediated by the ventromedial hypothalamic nucleus (VMN) [5,7], and silencing of ERα in this brain area results in diminution or suppression of the lordosis response in females rats and mice. To the contrary, the ERβ does not seem to be involved in female sexual behaviors [4,8,9].

In addition to their effects on female sexual behaviors, estrogens have anxiolytic properties in several standard tests, for example the elevated plus-maze [10], the light/dark choice procedure [11], or the open field [12]. These effects are usually attributed to the ERβ [12–15]

whereas the ERα is considered to promote anxiety. Silencing of the ERα decreased indicators of fear in a light/dark choice test [7] and an ERα agonist increased fear-potentiated startle [16], just to mention two examples. However, there are also reports of anxiolytic effects of the ERα [17]. The conflicting results could be reconciled by proposing that the ERα has a context-dependent, dual effect on anxiety, being anxiolytic in safe environments and anxiogenic in threatening ones [18].

In a previous study [1], we made a detailed description of the behavioral effects of an ERα- and an ERβ agonist in female rats living in a seminatural environment in which emotional challenges could be introduced. We found that the ERα agonist propyl-pyrazole-triol (PPT) increased fear reactions in threatening contexts (white noise and fox odor) only. The ERβ agonist diarylpropionitrile (DPN) had some anxiolytic effects in these contexts.

In our earlier study, the ER agonists were administered systemically, precluding any speculations as to their site of action. Now, we evaluate the role of the ERs in specific brain areas by silencing the expression of either the ERα or the ERβ with local administration of shRNA directed against each of these receptors. One target site was the VMN. The ERα within this nucleus is essential for female sexual behaviors and has been reported to be one site of action for the anxiogenic effects of this receptor [7,19]. It was originally reported that the VMN contains a large number of ERα but very few, if any, ERβ [20]. However, later studies revealed that the ERβ indeed is expressed in the VMN in adult animals, at least in the ventrolateral portion [21,22]. The behavioral function of this receptor within the VMN has not been evaluated. Even though it is unlikely that sexual behavior would be modified by silencing the ERβ, emotional responses to environmental disturbances might be modified.

The central amygdala (CeA) is the main source of output from the basolateral and medial amygdala [23] and has been found to be important for fear and anxiety responses [24,25]. It appears that corticotropin-releasing hormone(CRH)-containing neurons in this structure mediate these responses [26] in addition to their well-known role in physiological stress reactions [27]. The central amygdala expresses both ERα and ERβ, but the latter seems to be more abundant [20,28,29]. It has been reported that local administration of a glucocorticoid agonist into the CeA is anxiogenic, and that this response is reduced after systemic treatment with an ERβ agonist [30]. Even though these data do not show that the agonist acted within the CeA when reducing anxiety, it is possible to suggest that the ERβ within the CeA modulates anxiety responses. Furthermore, the enhanced expression of CRH in that area observed following systemic treatment with kainic acid is reduced by estradiol [31]. These findings show that neurons in the CeA are responsive to estradiol, perhaps resulting from the activation of the ERβ. Possible functions of the ERα in this structure remain unknown.

In order to evaluate the question of context-dependent responses to site-specific alterations in the activity of estrogen receptors, we exposed groups consisting of both male and female rats living in a seminatural environment to different emotion-inducing stimuli. Either the ERα or the ERβ was silenced in the VMN or the CeA. The emotion-inducing stimuli employed have previously been shown to elicit different behavioral responses, presumably associated with different emotions [1], These stimuli were lavender odor and chocolate flavored-food, known to produce a state of positive affect [32–34]. We also used white noise and fox odor in order to produce fear responses and an aversive emotional state [35,36]. Finally, a piece of music was played to the rat. The particular piece used here has been reported to produce estrogen-dependent anxiolysis on the elevated plus-maze and in the light-dark transition test [37], although we have found that it produces a slight fear reaction in the seminatural environment [1]. The potentially aversive properties of music were not known at the time the present experiment was run.

The proposed experiment would provide a picture of the potential importance of the estrogen receptors in the VMN and the CeA for emotional responses in safe as well as in threatening contexts in a procedure with external validity. Perhaps this could have some bearing on the issue of human sex differences in the prevalence of anxiety, depression and some other neuropsychiatric disorders [38,39].

2. Material and methods

2.1. Subjects

A total of 64 female and 48 male Wistar rats (200 g and 250 g respectively upon arrival) were obtained from Charles River (Sulzfeld, Germany). The rats were housed in same-sex pairs in standard cages (Macrolon IV, 43 × 26 x 15 cm, l x w x h) prior to the beginning of the experiment, with water and food (RM1, Special Diets Services, Witham, UK) available ad libitum. The ambient sound level averaged 40 dB due to the ventilation system; the temperature was maintained at 21 ± 1 °C, and the humidity to 55 ± 10%. The rats were submitted to an 12L:12D h reversed light cycle, lights being on between 11:00 pm and 11:00 am.

2.2. Surgery

Three weeks before the beginning of the experiment, the females underwent ovariectomy and stereotaxic surgery under anesthesia with a ketamine/xylazine cocktail (10 and 100 mg/kg, respectively). Immediately after ovariectomy, the females were fixed in a stereotaxic frame, and a small incision was made on top of the skull. Bilateral cannulae (30 gauge) were aimed at either the VMN (coordinates: anteroposterior: -2.56; mediolateral: ± 0.55; dorsoventral: -9.50) or the CeA (anteroposterior: -2.30; mediolateral: ± 4.00; dorsoventral: -7.00). Coordinates were based on the Paxinos and Watson atlas [40]. To silence estrogen receptors, we used a short hairpin RNA (shRNA) encoded with an adeno-associated viral (AAV) vector. The females were bilaterally infused with 1 μl of an AAV vector directed either against the ERα (AAV-ERα) or the ERβ (AAV-ERβ). Control animals received an AAV vector that encoded a firefly luciferase (AAV-luc). This vector does not affect the estrogen receptors. All vectors contained an independent enhanced green fluorescent protein (EGFP). The shRNAs against ERα as well as luciferase employed here have been previously described in detail [5]. The shRNA against ERβ has been described more recently [41]. Both the AAV-ERα and the AAV-ERβ have been shown to silence the intended receptor without affecting expression of the other. The infusion lasted 10 min (infusion rate: 0.10 μl/min) (Hamilton syringe and infusion pump), and the infusion cannulae were carefully withdrawn 10 min after the end of the infusion. Fentanyl, 0.05 mg/kg every 12 h, was provided for 72 h postoperatively.

2.3. Hormone treatment

Estrus was induced by sequential treatment with 17β-estradiol benzoate (EB, 18 μg/kg) followed by progesterone (P, 1 mg/rat) 48 h later (both were obtained from Sigma Aldrich, St Louis, MO), and dissolved in peanut oil (Den norske eterfabrikk, Norway). The hormones were injected subcutaneously in a volume of 1 ml/kg for EB and 0.2 ml/rat for P.

2.4. Apparatus

During the experiment, the rats were housed in a seminatural environment composed of an open area (210 × 120 × 80 cm, l × w × h) connected to a complex burrow system by 4 small openings (Fig. 1). The burrow system was maintained in the dark while the open area was submitted to the same light cycle as previously mentioned. The light intensity was 180 lx during the day and 30 lx during the night. Dawn and dusk were stimulated by 30-min light transitions. The ambient sound level, humidity and temperature were the same in both parts of the seminatural environment. The entire floor of the seminatural environment was covered with wood chips (Tapvei, Harjumaa, Estonia). Bedding material (Happi mat, Datesend, Manchester, UK) was provided in the nest boxes in the burrow. Wood sticks (Tapvei) were added as enrichment to the open area, and 3 red polycarbonate huts (Datesend, Manchester, UK) were disposed on the floor. Four bottles (1.5 l each) dispensed tap water in a corner of the open area and about 2 kg of the habitual food was put on the floor. Two nozzles in the walls were connected to an odor distribution system (Olfactory Stimulus Package, Medical Associates, Georgia, Vt) and produced a constant air stream of 3 l/min. One nozzle was located in the long, back tunnel in the burrow and the other in the far wall of the open area. In addition, a sound system consisting of two A60 stereo speakers (Creative, Clas Ohlson, Norway), one in each part of the environment, was installed. Two cameras fixed about 2 m above the floor filmed the entire experiment.
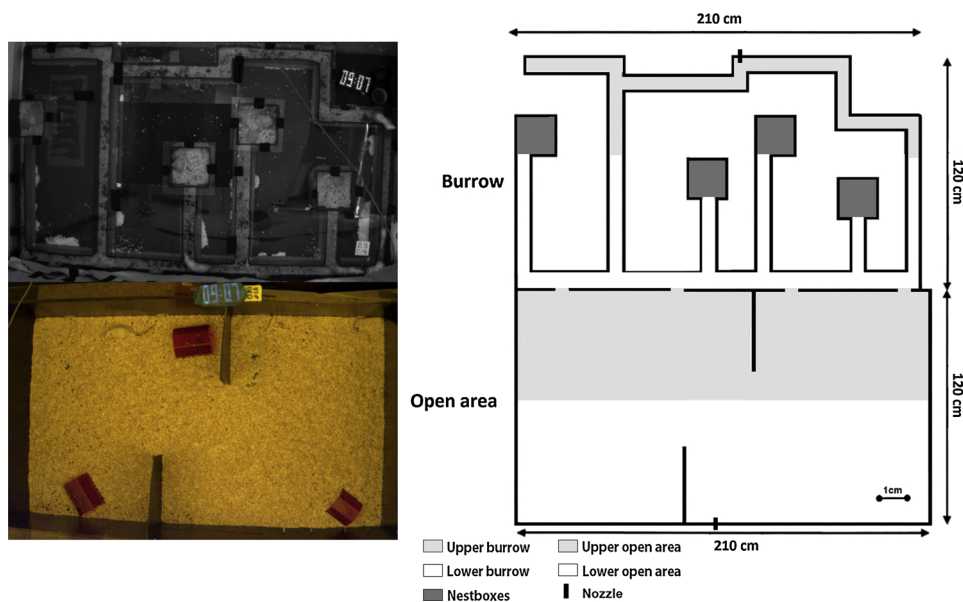
Fig. 1. (A) Picture of the seminatural environment. (B) Drawing of the seminatural environment and division in zones.

In the burrow, infrared lights (850 nm) allowed for video recording. The Media Recorder (Noldus, Wageningen, The Netherlands) was used for creating and storing the video files. This experimental setup has been described earlier [1,42–44].

2.5. Emotion-inducing stimulations

The rats were exposed to 5 experimental stimuli. Each of these stimuli have previously been shown to elicit different behavioral patterns, probably caused by different emotions [1]. The emotion-inducing stimuli chosen were either positive or negative to the rats, and stimulated different sensory modalities: olfactory, auditory or gustatory. The stimuli, in the order of presentation, were:

1 Lavender odor from 1.5 ml of *Lavandula angustifolia* essential oil (AromaBio, Lyon, France) replaced the room air stream through the nozzles (30 min). The odorant was put on a cotton pad in a glass jar. This stimulus has been reported to be anxiolytic in rats and humans [33,45].
2 Mozart's sonata for two pianos K448, played by Murray Perahia and Radu Lupu, recorded at Snape Maltings Concert Hall, Suffolk, England. CD from Sony Music Entertainment was played at 55–60 dB for 24 min and 18 s, the duration of the sonata. This Mozart piece has been found to be anxiolytic [46,47], particularly effective in proestrus females [37].
3 Thirty-five chocolate pellets (35 g) (Supreme Mini-Treats 1 mg; F05472;Bio Serv, Frenchtown, NJ) placed on a Petri dish (diameter 100 mm) in the middle of the open area for 30 min. Chocolate-flavored food is highly palatable for rats [32,34], and is known to produce positive affect [48].
4 White noise produced by a noise generator (Lafayette instruments, Lafayette, IN) at 90 dB for 7.5 min. This stimulus is routinely used for inducing strong fear reactions in rats [35,49].
5 Fox odor from 35 μl of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Contech, Delta, BC, Canada) for 30 min. The odor distribution system used to produce lavender odor was used also here. TMT odor produces fear reactions similar to those produced by exposure to a living predator [50,51].

2.6. Procedure

On day 0 at 9.00 a.m. the rats were weighed and shaved in different

patterns on the back. In addition, black marks were made on the tail. Thereby it was possible to identify the individuals on video. At 1.00 pm, the rats were released from their cages into the seminatural environment. On day 5 at 9.00 a.m. the females were captured and injected with EB. On day 7 at 9.00 a.m. the rats were captured again and injected with P. Four hours later, at 1.00 pm the sequence of emotional stimuli was initiated. There was a 50-min interval between the end of one stimulus and the start of the following.

The order of presentation of the emotional stimuli was kept constant throughout the experiment. The reason for this, as well as possible consequences, have been discussed in detail elsewhere [1]. Briefly, there are reasons for believing that the effects of the stimuli would have dissipated during the 50-minutes inter-stimuli interval. The exception is fox odor, which may cause behavioral alterations for several hours following exposure [50]. Therefore, this was the last stimulus to be applied.

It must also be pointed out that rats, in their natural habitat, are likely to be exposed to a sequence of events, both attractive and aversive, during the course of one single night. Thus, it can be maintained that the exposure to several kinds of stimulations used here increases the external validity of the procedure.

2.7. Behavioral observations

Observation of the females' behavior was limited to the last 15 min of lavender and fox odor exposure, when the odor should have full behavioral effects [52,53]. This was also the case for exposure to music. During the availability of chocolate, the first 15 min were observed. This made it possible to determine the immediate response to an attractive stimulus. Moreover, most of the chocolate was consumed during this period. The entire 7.5 min of noise exposure was observed. We also observed behavior during the 7.5 min period following the end of white noise. Possible treatment effects on the recovery of pre-noise behavior could thereby be established. Behavior was scored according to a slightly modified version of the ethogram used in a previous study [1] (Table 1), using the Observer XT 12.5 (Noldus, Wageningen, The Netherlands).

2.8. Design

Fifteen groups of 7 rats each (4 females and 3 males) were successively run in the seminatural environment. Thus, a total of 60 females

Table 1

Ethogram, definition of recorded behaviors. f = frequency; d = duration; l = latency; o = occurrence. [a], behavior observed only in the presence of chocolate. [b], behavior only observed during exposure to white noise.

Category	Behavior pattern	Definition
Female sexual behaviors	Lordosis; f,o	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.
Female attractivity	Mounts received; f	Male catches the female by her waist and puts his belly over her back, with pelvic thrusting.
Prosocial behaviors	Resting with another rat; f,d	Rests immobilized in relaxed position at a distance shorter than one rat to one or several females.
	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	The female faces a male, nose to nose, heads up, with or without boxing.
	Nose-off female; f,d	The female faces another female, nose to nose, heads up, with or without boxing.
	Flee from male; f	Escapes from agonistic interaction by running away or simply turning head away from a male.
	Flee from another female; f	Escapes from agonistic interaction by running away or simply turning head away from a female.
Exploratory behaviors and ambulatory activity	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.
	Sniffing the nozzles; f,d,l	Sniffs the nozzles in the wall distributing pure air of perfumed air (lavender or fox odor). The latency is the time between the beginning of the observation and the first episode of nozzle sniffing.
	Transitions; f	Displays a behavior in a zone different from the one in which the previous behavior was displayed.
	Approach to chocolate[a]; f,l	Comes close enough for making snout or paw contact with the chocolate pellets. The latency is the time between putting the petri dish on the floor of the open area and the first approach.
	Grabbing[a]; f	Grabs chocolate with paws or mouth.
	Eating chocolate[a]; f,d	Chews on chocolate.
Non-social and maintenance behaviors	Resting alone; f,d	Rests immobilized in relaxed position at a distance longer than one rat to a conspecific.
	Drinking; f,d	Self explanatory.
	Eating regular food; f.d	Self-explanatory.
	Selfgrooming and scratching; f,d	Self explanatory.
Fear- and anxiety-related behaviors	Hide alone[b]; f,d	Immobilized in a corner or nest box at a distance longer than one body length to another rat.
	Huddling[b]; f,d	Immobilized in a corner or in a nest box in close contact with one or several other rats.
	Freezing[b]; f,d	Immobilized in rigid position without any movement including those of vibrissa.
	Startle[b]; o	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.
	Flee from noise[b]; o,l	Rushes into the burrows at the onset of the white noise. The latency is the time from onset of the noise until the rat escapes from the open field into the burrow.
	Risk assessment; f,d	Approaches the openings in the burrow and observing the open area. A rat is considered in the opening as long as a body part between the muzzles and the butt remains in the door.

participated in the experiment. They were divided in six treatment groups of 10 females each: (1) AAV-luc VMN; (2) AAV-ERα VMN; (3) AAV-ERβ VMN; (4) AAV-luc CeA; (5) AAV-ERα CeA; (6) AAV-ERβ CeA. In each group of the seminatural environment, all females had different treatments. Apart from this, the 6 treatments were randomly distributed among the 15 groups of rats run in the seminatural environment.

2.9. Immunocytochemistry

The day after the experiment was terminated, the animals were euthanized with an overdose of pentobarbital. They were perfused with PBS followed by 4% paraformaldehyde. We removed the brain and stored it in paraformaldehyde at $+4\,°C$ overnight. The following day, the brains were transferred to 10% sucrose in PBS, the subsequent day to 20% sucrose in PBS and the third day to 30% sucrose in PBS, where they were kept for seven days. The brains were then frozen in isopentane cooled on dry ice, and stored at $-80\,°C$ until processing. The brains were frozen-sectioned in 40 µm slices with a sledge microtome (SM2000R, Leica Microsystems Nussloch, Germany), and the VMN and the CeA sections were collected and processed in accordance with a conventional free-floating protocol.

Two sessions of immunocytochemistry were run, one for the brains that had received AAV-ERα, and one for the brains that had received AAV-ERβ. The appropriate sections were treated with antibodies against ERα (c1355, polyclonal, 1:25000, Merk Millipore, Germany) and EGFP (ab6673, GFP, 1:5000, Abcam, Cambridge, MA) in

combinations with secondary antibodies (BA1000, biotinylated rabbit, Vector laboratories, Burlingame, CA) and avidin-biotin peroxidase complex (PK-6101, ABC elite kit from Vector laboratories, Burlingame, CA) to identify cells containing ERα, and injection localization, respectively. After antibody reactions and several washings in PBS, sections were stained with diaminobenzidine (DAB). DAB revealed injection localization by brown coloration of EGFP while the ERα was colored in dark purple by the addition of nickel. For the second session, the appropriate sections were treated with antibodies against ERβ (PA1-310B, polyclonal, 1:1000, ThermoFisher Scientific, San Jose, CA) and EGFP (ab290, 1:1000, Abcam, Cambridge, MA). The PA1-310B does not bind to the ERα [54], and it has been used to quantify ERβ expression in many studies [17,55,56]. The same secondary antibodies and avidin-biotin peroxidase complex were used as previously. After antibody reactions and washing in PBS, sections were stained with DAB, revealing the injection localization in brown coloration, and ERβ in blue, by the addition of cobalt chloride.

For counting purposes, microphotographs of the stained sections were taken using an Axiophot photomicroscope (Carl Zeiss, Obercochen, Germany) connected to a digital camera (Nikon DS, Nikon, Tokyo, Japan). Then, the pictures were transferred to a computer and opened with Photoshop (Adobe Photoshop CS6). We selected three sections per individual and manually counted the density of ERs (number of ER/mm²) by dividing the number of stained cells counted by the surface of each nucleus.

2.10. Data preparation and statistical analysis

We recorded the time spent in the burrow system and the open area, as well as the frequency of transitions between the zones of the semi-natural environment (Fig. 1B). The frequency and, whenever possible, total duration of each behavior displayed was determined. Then we evaluated the stability of behavior during the observation period. We randomly picked some of the behaviors described in the ethogram (Table 1), and compared their frequency and duration in the first and last minute of observation with paired t-test. For example, nose-off frequency and duration were stable across the observation period, both for females infused in the VMN and the CeA ($p > 0.446$). Likewise, frequency and duration of paracopulatory behaviors ($p > 0.357$), sniffing another rat ($p > 0.537$) or self-grooming ($p > 0.083$) were stable across the observation. Therefore, the raw data for each behavior was converted into number per minute and duration per minute of observation. This made it possible to compare stimuli with different length of observation period.

The aim of some comparisons was to determine whether behavior during one stimulus indeed differed from the behavior during the others. To that end, we compared the target stimulus to the mean of the four other stimuli. This was done by calculating a difference quotient in the following way: difference quotient = [(target stimulus - mean of the four other stimuli)/mean of the four other stimuli]. If behavior during exposure to the target stimulus was identical to the mean of the other stimuli, the difference quotient would be zero. The larger the deviation from zero, the larger the effect of the stimulus compared to the other stimuli. Data are presented as the difference quotient. This procedure has been used earlier to determine the effect of emotion-inducing stimuli on behavior [42]. In order to avoid any potential confounding effect of the treatment, this analysis was based exclusively on frequency data from the rats treated with AAV-luc in the VMN and the CeA. Since the differences between stimulus effect on behavior frequency and duration were marginal, we present only the difference in behavior frequency, since this parameter was available for all behaviors. Only the time spent in the burrow, in the open area, and in the openings (i.e. risk assessment) are expressed as durations. In addition, data collected during the last 50 s of white noise exposure as well as for the 7.5 min directly following white noise offset were divided into 10 intervals of 50 s each. This allowed for an analysis of the behavioral recovery following the end of white noise exposure.

The number of ERα and ERβ in the females infused with vectors directed against these receptors, in the VMN and the CeA, was compared to their respective controls (AAV-luc VMN and AAV-luc CeA, respectively) with the t-test for independent samples.

To assess if behavior during a specific stimulus differed from the mean of the other stimuli, we submitted the difference quotient to a one-sample t-test comparing the obtained value to 0. The p-value was adjusted with the Bonferroni correction to the 5 comparisons made, corresponding to the 5 stimuli. When the use of the one-sample t-test was not possible because of non-normal data distribution according to the Shapiro-Wilk test, we used the Wilcoxon one-sample test, and the Bonferroni correction.

For the evaluation of the effects of gene silencing, the data concerning the VMN and the CeA were analyzed separately. For these analyses, both behavior frequency and duration were considered. When possible, we used two-way ANOVA with stimulus as within-groups factor and treatment as between-groups factor, followed by the Tukey HSD post hoc test. In case of significant interaction between treatment and stimulus, simple main effects were analyzed.

When the data deviated from the normal distribution according to the Shapiro-Wilk test, we analyzed the effect of the treatment with Kruskal-Wallis ANOVA, followed by the Conover post hoc test. Finally, the probabilities to display lordosis and to flee the white noise at its onset were analyzed with the binomial test, which p-value was adjusted to the two comparisons made (AAV-ERα to AAV-luc, and AAV-ERβ to AAV-luc).

To determine behavioral recovery after white noise, data from the last 50 s of white noise exposure until 7.5 min after its offset were analyzed using a mixed two-way ANOVA with time interval as within-groups factor and treatment as between-groups factor, followed by the Tukey HSD test. Simple main effects were analyzed after significant interaction between treatment and time interval. When the data deviated from the normal distribution according to the Shapiro-Wilk test, we analyzed the effect of treatment with Kruskal-Wallis ANOVA and the effect of time intervals with Friedman's ANOVA. In case of significance, post hoc differences were analyzed with the Conover test. Only differences from the last 50 s of white noise exposure are reported.

The significance threshold was P < 0.05. Statistical analyses were conducted with IBM SPSS Statistics, version 24 and R, version 3.4.3 (core and PMCMRplus packages).

2.11. Co-occurrence analysis

The seminatural environment allows the rat to express a substantial amount of their behavioral repertoire. The resulting behavioral observation produced a list of behaviors in chronological order, for each individual observed. Using a moving window of 4 behavioral items, we determined how often one behavior item occurred together with another in the same window. This was defined as a co-occurrence. Based on the relative frequency of co-occurrences of one behavior together with each of the other behaviors, clusters of significantly co-occurring items could be established as statistically independent profiles of items [57]. Descending hierarchical classification determined the probability, as evaluated by χ^2 analysis, for an item to be more present in one cluster than in any of the other clusters [58,59]. Co-occurrence clusters were visualized using the Fruchterman-Reingold algorithm, with the Iramuteq software (Interface de R pour les Analyses Multidimensionnelles de Textes et Questionnaires, available at http://www.Iramuteq.org/). This procedure has been found to offer valuable information concerning the structure of behavior, and it has been extensively described elsewhere [1,42]. This analysis could be based either on the entire data set, or on data obtained during a particular emotion-inducing stimulus and/or from animals receiving a particular treatment.

3. Results

3.1. Histology

Females with a reduction of the number of targeted receptor of more than 80% with respect to the appropriate control were included in the analyses. A low reduction could have resulted from a misplaced cannula, or a low viral transduction in the target area. Forty-four females satisfied the criterion of a 80% reduction minimum. The location of the infusion site is shown in Fig. 2. The slices from two females treated with AAV-ERα in the VMN were of poor quality and ICC were not performed. However, none of these females responded with lordosis to the males' mounts. We have previously reported that this behavioral response is a biomarker of a substantial reduction of the number of ERα in the VMN [19,60,61]. Therefore, we included these females in the AAV-ERα VMN group. The females were distributed as follows: AAV-luc-VMN n = 10; AAV-ERα-VMN n = 7; AAV-ERβ-VMN n = 6; AAV-luc-CeA n = 10; AAV-ERα-CeA n = 7; AAV-ERβ-CeA n = 6. In the included females, we observed a reduction of 94% in the number of ERα in the CeA ($t_{(9)} = 8.98$, $p = 0.011$) and a reduction of 95% of ERα in the VMN ($t_{(13)} = 14.13$, $p < 0.001$) (Fig. 3A). For ERβ, we achieved a 83% reduction in the CeA ($t_{(9)} = 16.12$, $p < 0.001$) and a 84% reduction in the VMN ($t_{(8)} = 12.79$, $p = 0.001$) (Fig. 3B).
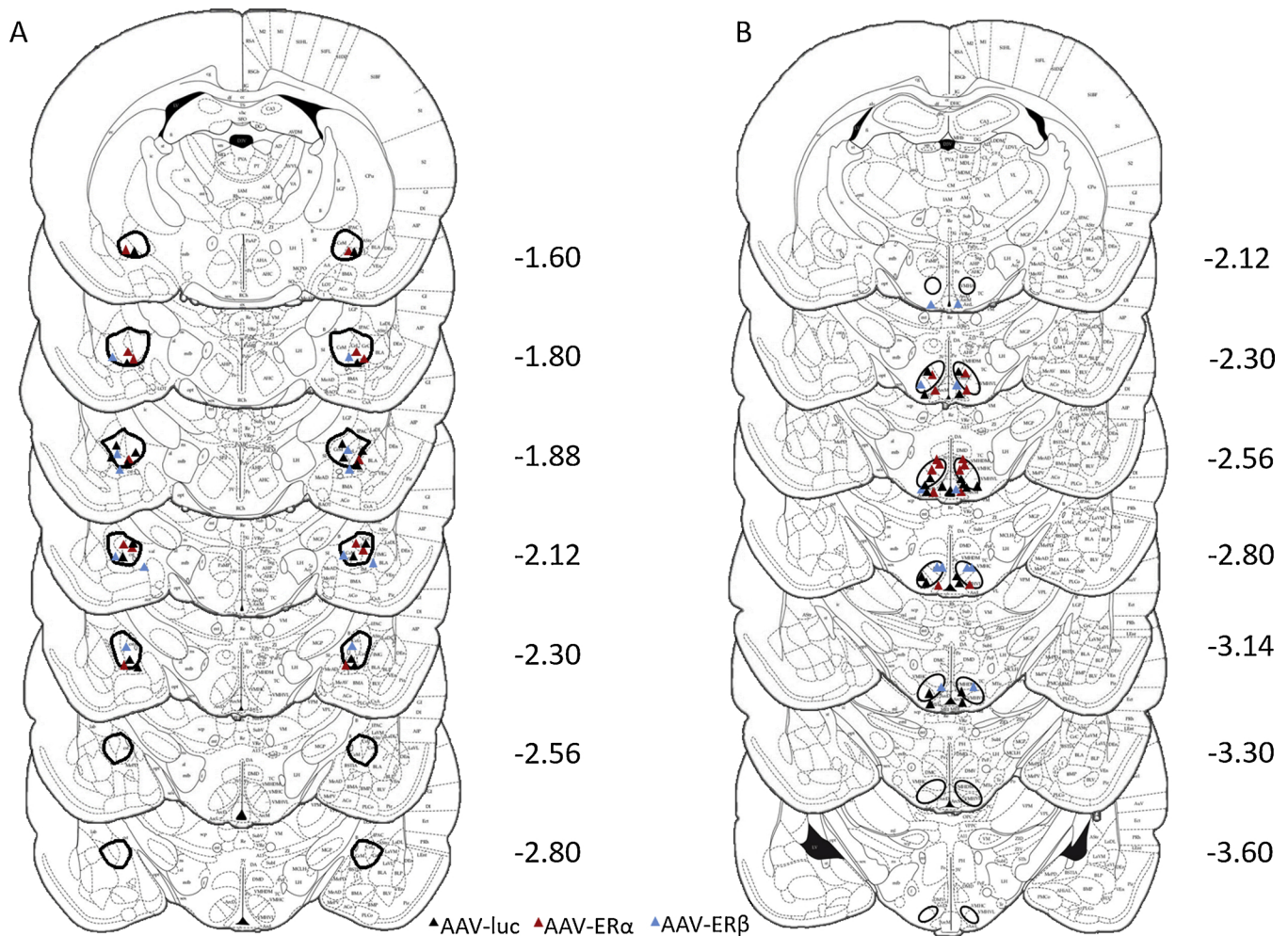
Fig. 2. Infusion sites within the central amygdala (CeA; Panel A) and the ventromedial nucleus of the hypothalamus (VMN; Panel B) with shRNA directed against the ERα (AAV-ERα; in red), ERβ (AAV-ERβ; in blue) or luciferase (AAV-luc; in black). Numbers to the right represent distance (in mm) from bregma.

3.2. Effect of the emotional stimuli (Table 2)

One-sample t-tests were used to determine whether the difference quotient obtained for each of the recorded behaviors during each emotion-inducing stimulus differed from 0. Only animals infused with AAV-luc were used, and the CeA and the VMN groups were pooled. Exposure to lavender odor increased the transitions in the open area ($t_{(19)} = 3.666$, p = 0.008) and decreased the time spent in the burrow ($t_{(19)} = 2.873$, p = 0.049). In addition, females sniffed the males more frequently during this stimulus ($t_{(19)} = 3.355$, p = 0.017), but nosed-off other females less frequently ($t_{(19)} = 3.220$, p = 0.023). We found no other significant effect of the lavender odor compared to the mean of the other stimuli (all p's > 0.084) (Table 2).

During exposure to music, the females displayed less transitions between the zones in the burrow ($t_{(19)} = 5.016$, p < 0.001), and decreased duration of risk assessment ($t_{(19)} = 4.007$, p = 0.004). Furthermore, the exploratory behaviors sniffing the floor ($t_{(19)} = 6.495$, p < 0.001) and rearing ($t_{(19)} = 5.199$, p < 0.001) were decreased during music exposure. We observed a diminution of the rejection frequency ($V_{(19)} = 2.620$, p = 0.045), and of the antisocial behaviors nose-off to another female ($t_{(19)} = 3.245$, p = 0.021) and fleeing from another female ($t_{(19)} = 5.671$, p < 0.001). The frequency of the pro-social behavior resting with another rat also decreased ($V_{(19)} = 2.703$, p = 0.035). The other observed behaviors did not differ from the mean of the other stimuli (all p's > 0.055) (Table 2).

During exposure to chocolate, the time spent in the burrow was

strongly decreased ($t_{(19)} = 55.587$, p < 0.001). The frequency of resting with another rat was also significantly decreased ($V_{(19)} = 2.703$, p = 0.035), but the other behaviors were not modified (all p's > 0.106) (Table 2).

During exposure to white noise, the number of transitions in the burrow, as well as the time spent in the burrow, were increased (respectively: $t_{(19)} = 4.181$, p = 0.003; $t_{(19)} = 5.005$, p < 0.001). The number of transitions in the open area and the time spent in the open area were reduced (respectively: $t_{(19)} = 30.853$, p < 0.001; $t_{(19)} = 138.883$, p < 0.001). The exploratory behavior sniffing the floor was increased ($t_{(19)} = 7.098$, p < 0.001), whereas the prosocial behavior resting with another rat ($V_{(19)} = 4.472$, p < 0.001), as well as the non-social behaviors resting alone ($V_{(19)} = 4.472$, p < 0.001) and drinking ($V_{(19)} = 4.472$, p < 0.001) were suppressed. Most sexual behaviors were strongly inhibited (paracopulatory behaviors: $V_{(19)} = 4.472$, p < 0.001; lordosis: $V_{(19)} = 4.337$, p < 0.001; LQ: $V_{(19)} = 3.545$, p < 0.001; rejection: $V_{(19)} = 2.397$, p = 0.045). Finally, the anti-social behaviors nose-off and fleeing from other females were increased (respectively: $t_{(19)} = 4.861$, p = 0.001; $t_{(19)} = 3.435$, p = 0.014). We observed no difference in these behaviors when they were directed to males (p > 0.161). The remaining behaviors were not significantly impacted by white noise (all p's > 0.053) (Table 2).

Finally, during exposure to fox odor, the number of transitions was decreased both in the open area ($t_{(19)} = 3.133$, p = 0.027) and in the burrow ($t_{(19)} = 5.016$, p < 0.001). However, the time spent in the
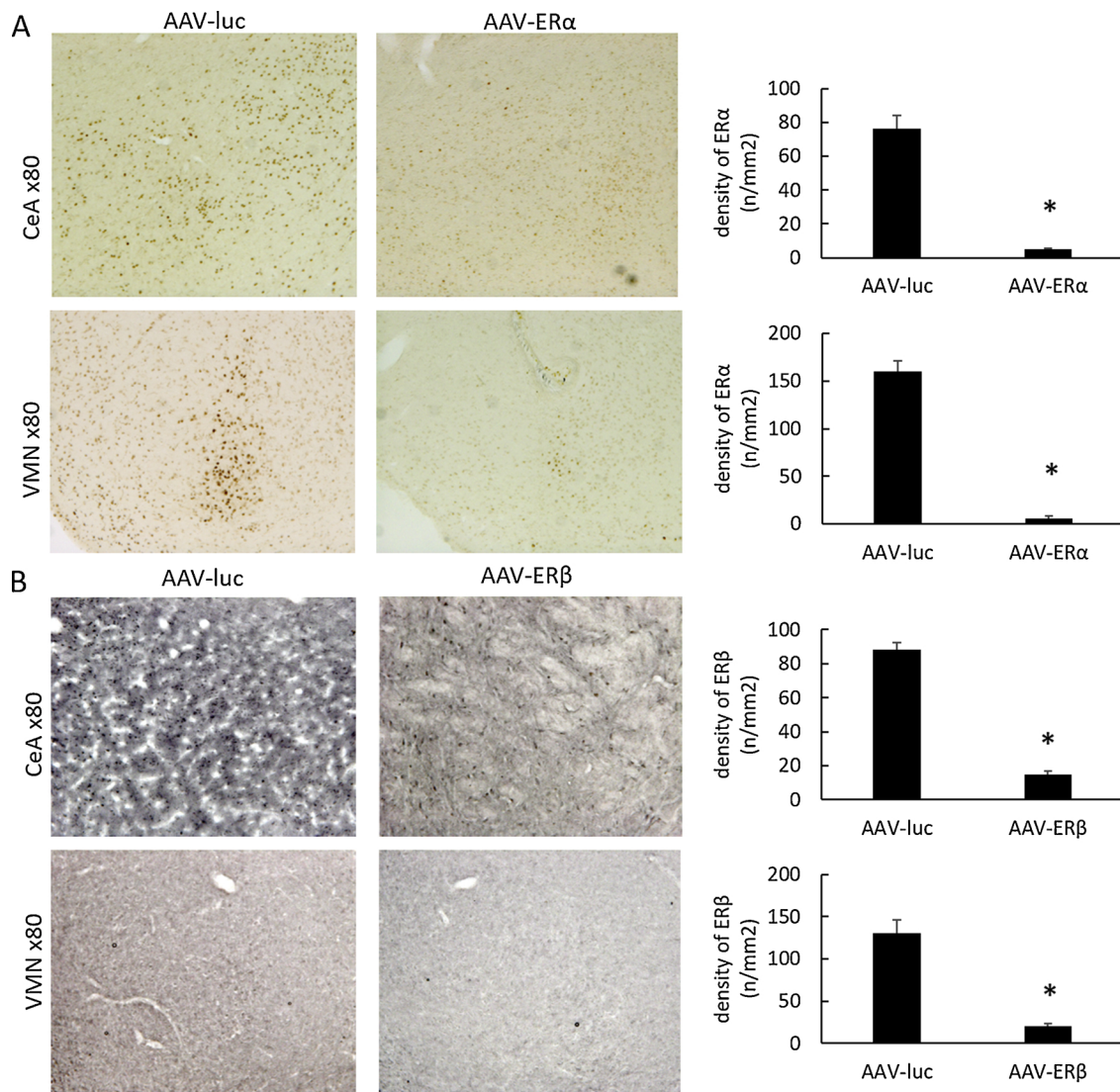
Fig. 3. Immunocytochemical staining of estrogen receptor expression in brain slices in the CeA and the VMN after bilateral infusion of (A) AAV-ERα or (B) AAV-ERβ. The panels to the far left show pictures from control females treated with AAV-luc. The figures to the right show the density of ERs. Data are mean + SEM. *, different from AAV-luc.

burrow was increased ($t_{(19)} = 6.509$, p < 0.001). The frequency of sniffing the floor and rearing were decreased (respectively: $t_{(19)} = 2.875$, p = 0.049; $t_{(19)} = 6.850$ p < 0.001), and so was the rejection frequency ($V_{(19)} = 2.397$, p = 0.035). Most social behaviors were reduced by exposure to fox odor (sniffing another female: $t_{(19)} = 7.131$, p < 0.001; sniffing males: $t_{(19)} = 5.173$, p < 0.001; nose-off to another female: $t_{(19)} = 4.724$, p = 0.001; nose-off to males: $t_{(19)} = 7.724$, p < 0.001; fleeing another female: $t_{(19)} = 7.142$, p < 0.001; fleeing males: $t_{(19)} = 6.670$, p < 0.001). To the contrary, the frequency of resting alone increased ($t_{(19)} = 3.255$, p = 0.021). The other behavioral modifications did not reach significance (all p's > 0.075) (Table 2).

3.3. Effect of treatment in response to emotion-inducing stimuli

3.3.1. Effect of treatment on sexual behavior

Sexual behaviors deviated from the normal distribution according to Shapiro-Wilk's test. Therefore, the effects of treatment on these behaviors were analyzed with the non-parametric Kruskal-Wallis ANOVA. We found no effect AAV-ERα or AAV-ERβ infusion in the CeA on sexual behaviors (all p's > 0.060).

In the VMN, females belonging to the AAV-ERα group had a lower probability to display lordosis than females from the control group, all emotion-inducing stimuli collapsed (binomial test, p = 0.024) (Fig. 4A). When looking at the specific emotion-inducing stimuli, treatment with AAV-ERα reduced the probability to display a lordosis during exposure to lavender (binomial test, p = 0.038) and chocolate (binomial test, p = 0.038), but not during exposure to music, white noise and fox odor (all p's > 0.45) (Fig. 4C). Despite the reduction in the probability to display lordosis, the lordosis frequency itself was not significantly reduced ($\chi^2_{, N=23} = 2.339$, p = 0.310) (Fig. 4B). Similarly, the decrease in mounts received and paracopulatory behaviors did not reach significance (all p's > 0.350). Likewise, the lordosis quotient and the rejection frequency were not affected by treatment (all p's > 0.819) (data not shown).

3.3.2. Effect of treatment on pro- and anti-social behavior

We did not find any effect of AAV-ERα nor AAV-ERβ infusion in the CeA on pro- and antisocial behaviors, whether directed to males or to other females (all p's > 0.076) (data not shown). No effect on these behaviors was obtained when females were infused in the VMN (all p's > 0.130) (data not shown).

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Table 2
Effects of the stimuli. Results are indicated as difference quotient compared to the mean of the four other stimuli (mean ± SEM). Significant differences are indicated in **bold italic**, * p < 0.05.

|		Lavender odor	Music	Chocolate	White noise	Fox odor
Sexual behaviors	Paracopulatory behaviors	+1.81 ± 0.69	−0.02 ± 0.42	+0.54 ± 0.44	***−1.00 ± 0.00****	−0.52 ± 0.28
	Rejection	+2.25 ± 2.15	***−0.54 ± 0.27****	+0.57 ± 0.90	***−0.53 ± 0.27****	***−0.73 ± 0.18****
	Lordosis	+0.62 ± 0.65	−0.06 ± 0.47	+0.73 ± 0.64	***−0.92 ± 0.08****	−0.01 ± 0.58
	LQ	+1.20 ± 0.52	−0.21 ± 0.37	+0.77 ± 0.45	***−0.87 ± 0.13****	−0.34 ± 0.31
Prosocial behaviors	Sniffing males	***+1.33 ± 0.40****	−0.40 ± 0.22	+0.37 ± 0.27	−0.27 ± 0.10	***−0.62 ± 0.12****
	Sniffing other females	+0.32 ± 0.25	−0.36 ± 0.30	+0.98 ± 0.39	+0.06 ± 0.16	***−0.68 ± 0.10****
	Resting with another rat	+1.15 ± 1.28	***−0.56 ± 0.31****	***−0.56 + 0.31****	***−1.00 ± 0.00****	+2.27 ± 1.94
Antisocial behaviors	Nose-off males	+1.07 ± 0.48	−0.49 ± 0.17	+0.01 ± 0.34	+0.72 ± 0.31	***−0.81 ± 0.10****
	Nose-off other females	***−0.44 ± 0.14****	***−0.52 ± 0.16****	0.16 ± 0.27	***+2.62 ± 0.54****	***−0.69 ± 0.15****
	Fleeing males	+1.21 ± 0.67	−0.49 ± 0.21	+0.03 ± 0.33	+0.50 ± 0.31	***−0.78 ± 0.12****
	Fleeing other females	+0.28 ± 0.31	***−0.71 ± 0.12****	+0.15 ± 0.25	***+1.76 ± 0.51****	***−0.75 ± 0.11****
Exploratory behaviors and locomotory activity	Transitions in the burrow	−0.17 ± 0.17	***−0.55 ± 0.11****	+0.18 ± 0.21	***+1.70 ± 0.41****	***−0.57 ± 0.10****
	Transitons in the open area	***+1.30 ± 0.35****	−0.13 ± 0.23	+0.99 ± 0.44	***−0.93 ± 0.03****	***−0.52 ± 0.17****
	Time in the burrow	***−0.38 ± 0.13****	+0.28 ± 0.21	***−0.94 ± 0.02****	***+0.41 ± 0.08****	***+1.15 ± 0.18****
	Time in the open area	+0.76 ± 0.29	+0.50 ± 0.47	+0.42 ± 0.38	***−0.99 ± 0.01****	−0.25 ± 0.30
	Sniffing the floor	+0.16 ± 0.13	***−0.59 ± 0.09****	−0.21 ± 0.12	***+1.56 ± 0.22****	***−0.41 ± 0.14****
	Rearing	+1.43 ± 0.61	***−0.66 ± 0.13****	−0.40 ± 0.16	+1.23 ± 0.56	***−0.77 ± 0.11****
Non-social and maintenance behaviors	Resting alone	−0.10 ± 0.21	+0.76 ± 0.33	−0.05 ± 0.15	***−1.00 ± 0.00****	***+0.87 ± 0.27****
	Drinking	+2.46 ± 0.94	+0.25 ± 0.48	−0.09 ± 0.31	***−1.00 ± 0.00****	−0.49 ± 0.24
	Eating food	−0.47 ± 0.18	−0.41 ± 0.24	+0.46 ± 0.53	+0.55 ± 0.98	+0.06 ± 0.37
	Self-grooming	+0.20 ± 0.19	+0.00 ± 0.24	−0.17 ± 0.19	+0.05 ± 0.12	−0.06 ± 0.19
Fear-and anxiety-related behavior	Risk assessment	+0.07 ± 0.31	***−0.52 ± 0.13****	+0.52 ± 0.46	+0.28 ± 0.48	−0.20 ± 0.39
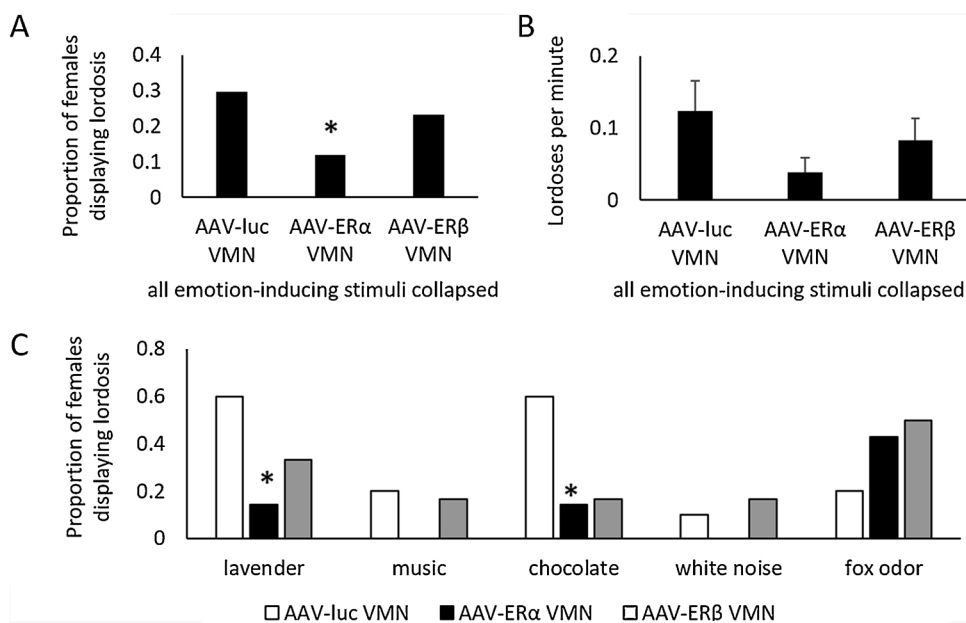
Fig. 4. (A) Proportion of females infused with shRNA directed against the ERα or the ERβ in the VMN displaying lordosis, all emotion-inducing stimuli collapsed; (B) Lordosis frequency in these females, all emotion-inducing stimuli collapsed, mean + SEM; (C) Proportion of females displaying lordosis at each of the emotion-inducing stimuli. *, different from AAV-luc.

3.3.3. Effect of treatment on exploratory behavior

Treatment in the CeA influenced olfactory exploration of the semi-natural environment according to the two-way ANOVAs for repeated measures (emotion-inducing stimulus x treatment), all observation collapsed. We found a main effect of treatment on the duration of sniffing the floor ($F_{2,20}$ = 3.787, p = 0.040). Females infused with AAV-ERβ spent more time sniffing the floor than the controls (p = 0.032) (Fig. 5A). We also found an interaction between treatment and stimulus in the duration of sniffing the floor ($F_{8,80}$ = 6.125, p = 0.025). Analysis of simple main effects within each stimulus showed an effect of lavender exposure ($F_{2,20}$ = 8.420, p = 0.002). During exposure to lavender odor, females infused with AAV-ERβ spent more time sniffing the floor than the controls (p = 0.010) (Fig. 5B). No other significant interaction between treatment and stimulus was found (all p's > 0.200). Behaviors specific to chocolate exposure (sniffing, eating, grabbing) and olfactory exploration (sniffing the nozzles during lavender or fox odor) showed no effect of treatment (all p's > 0.221).

In the VMN, treatment modified the rearing frequency ($F_{2,20}$ = 1.598, p = 0.030), but post hoc tests did not reach significance (all p's > 0.052) (data not shown). Treatment in the VMN did not modify chocolate-specific behaviors nor sniffing the nozzles (all p's > 0.610).

3.3.4. Effect of treatment on fear- and anxiety-related behavior

ANOVA of the data from females treated with the viral vectors in the CeA showed that the duration of risk assessment was modified by the treatment ($F_{2,20}$ = 4.150, p = 0.031). Females treated with the AAV-ERβ spent more time displaying risk assessment than the controls (p = 0.027) (Fig. 6A). However, anxiety-related behaviors specific to white noise (freezing, hiding alone, huddling, startle, flight) showed no influence of treatment (all p's > 0.304).

For females infused in the VMN, a behavior specific to white noise exposure, huddling, showed a treatment effect ($F_{2,20}$ = 7.914, p = 0.003;). Females treated with AAV-ERα had a reduced frequency of
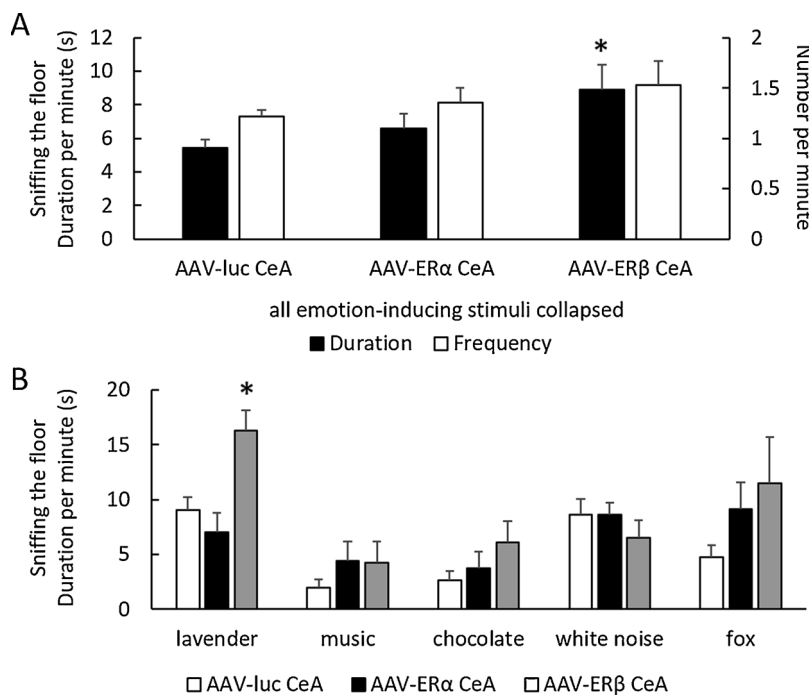
135

Fig. 5. (A) Frequency and duration of sniffing the floor in females infused with shRNA directed against the ERα or the ERβ in the CeA, all emotion-inducing stimuli collapsed; (B) Duration of sniffing the floor in these females at each of the emotion-inducing stimuli, data are mean + SEM. *, different from AAV-luc.

huddling compared to controls (p = 0.002) (Fig. 6B). The viral vectors did not modify other anxiety-related or white noise-specific behaviors (all p's > 0.065).

3.3.5. Effect of treatment on non-social, maintenance behaviors

Treatment in the CeA modified the frequency of eating ($H_{2,\,N=21}$ = 5.999, p = 0.050). Females treated with the AAV-ERβ ate food less often than the controls (p = 0.016) (Fig. 6C). No effect of treatment was found on the behaviors drinking, resting or self-grooming (all p's > 0.076). We did not find any effect of infusion in the VMN on these behaviors (all p's > 0.279).

3.3.6. Co-occurrence analysis of behavior in the CeA groups (Fig. 6)

AAV-ERα, AAV-ERβ and AAV-luc appeared in distinct clusters at each emotion-inducing stimuli except white noise. AAV-luc was mostly associated with the non-social behaviors drinking, eating food and self-grooming, and the exploratory behavior rearing during exposure to all the emotion-inducing stimuli. The cluster containing AAV-ERα included the sexual behaviors during exposure to lavender and fox odor (Fig. 7A-E). AAV-ERβ was associated with risk assessment during

exposure to lavender and music, and sniffing the nozzles during these two stimuli as well as during exposure to chocolate and white noise. During exposure to chocolate, AAV-ERβ was associated with most chocolate-specific behaviors (Fig. 7C). Only during exposure to white noise, AAV-ERα and AAV-ERβ appeared in the same cluster, together with fear-related behaviors hiding alone and fleeing the noise (Fig.7D.).

3.3.7. Co-occurrence analysis of behavior in the VMN groups (Fig. 8)

AAV-luc was consistently associated with the non-social behavior resting alone, and was associated with sexual behaviors at each stimulus except fox odor. During noise, AAV-luc was found in the same cluster as noise-specific behavior huddling (Fig. 8D). AAV-ERα was associated with rejection at all stimuli except chocolate (Fig. 8C). During fox odor, AAV-ERα and AAV-luc appeared in the same cluster associated with exploratory behaviors (Fig. 8E). AAV-ERβ appeared in the same cluster as AAV-ERα during exposure to lavender odor and music (Fig. 8A-B). AAV-ERβ was associated with risk assessment during lavender odor, music and white noise. During exposure to white noise and fox odor, AAV-ERβ was found in the same cluster as the anti-social behaviors nose-off and fleeing from another rat (Fig. 8D-E).
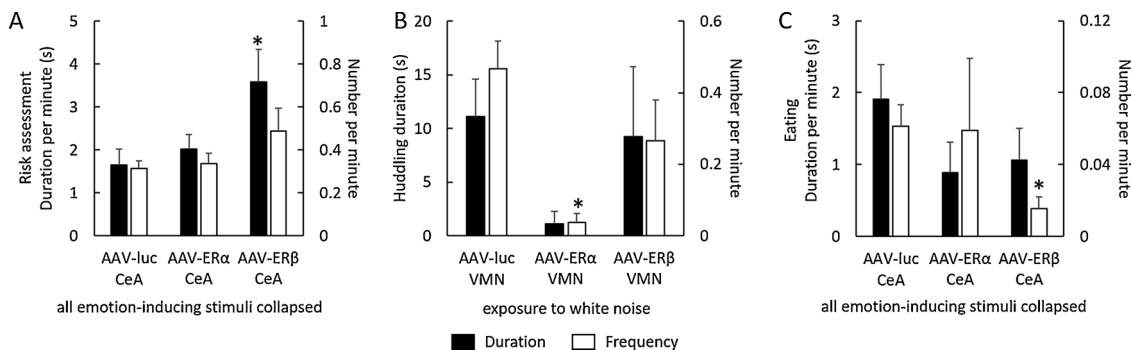

Fig. 6. (A) Frequency and duration of risk assessment in females infused with shRNA directed against the ERα or the ERβ in the CeA, all emotion-inducing stimuli collapsed; (B) Frequency and duration of huddling during exposure to white noise, in females infused with shRNA directed against the ERα or the ERβ in the VMN (C) Frequency and duration of eating over the entire observation period, in females infused with shRNA directed against the ERα or the ERβ in the CeA, data are mean + SEM. *, different from AAV-luc.
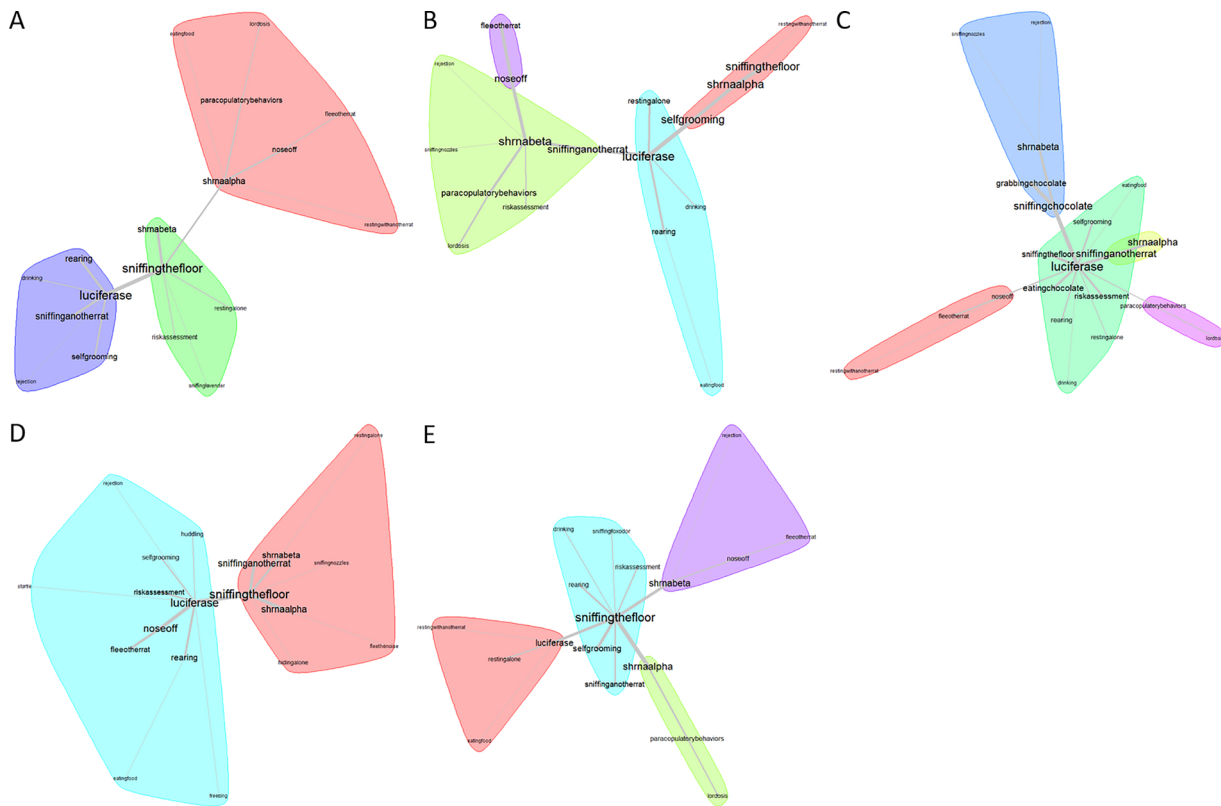
Fig. 7. Co-occurrence analysis showing main behavioral associations typical of each of the treatments in the CeA (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ), during exposure to (A) lavender odor, (B) music, (C) chocolate, (D) white noise and (E) fox odor (TMT). Clusters of behavioral association are represented in halos of different colors. 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.

3.4. Effects of ER knockdown on recovery from white noise

White noise caused numerous alterations in the females' behavior, as described above. Even though the viral vectors only affected huddling during this stimulus, it is possible that the recovery from the treatment-independent effects indeed could be affected by the treatment.

3.4.1. Central amygdala

When data satisfied normal distribution criteria according to Shapiro-Wilk's test, and when the error variances were homogenous according to Hartley's Fmax test, two way ANOVAs on one factor (time interval) and independent measures on the other (treatment) were performed. We did not find any main effect of treatment on behavioral changes after exposure to white noise (all p's > 0.077). Furthermore, ANOVAs did not find any interaction between treatment and time intervals (all p's > 0.101). For behavior not satisfying criteria for parametric analysis, Friedman's ANOVA found an effect of time intervals on the frequency of nose-off to other females ($\chi^2_{df=9} = 19.049$, p = 0.025), as well as on the frequency and duration of paracopulatory behaviors (frequency: $\chi^2_{df=9} = 21.675$, p = 0.010, duration: $\chi^2_{df=9} = 21.116$, p = 0.012). However, none of the post hoc tests for non-parametric analyses reached significance (all p's > 0.186) (data not shown). The modification of the time spent in the burrow and the open area was not significant (burrow: $F_{2,9} = 0.937$, p = 0.497; open area: $F_{2,9} = 1.163$, p = 0.328) (data not shown).

3.4.2. Ventromedial nucleus of the hypothalamus

Behavioral data of females infused in the VMN were analyzed with the same methods as that of females infused in the CeA. For females infused in the VMN, we found an effect of treatment on huddling during

the period of recovery from white noise (frequency: $H_{2, N=23} = 8.750$, p = 0.013; duration: $H_{2, N=23} = 8.591$, p = 0.014). Females treated with AAV-ERα had a reduced frequency (p = 0.006) and duration (p = 0.006) of huddling compared to controls (Fig. 9A). Analysis of treatment effect at each time interval showed that both AAV-ERα and AAV-ERβ groups had a lower huddling frequency than controls during white noise exposure (AAV-ERα- AAV-luc, p < 0.001; AAV-ERβ- AAV-luc, p = 0.006) (Fig. 9B). Only the AAV-ERα group differed from controls in the duration of huddling. Females treated with AAV-ERα spent less time huddling than the control during the last interval of white noise exposure (p = 0.001) and the first interval after white noise offset (p = 0.011) (Fig. 9B). In addition, time intervals influenced the huddling frequency ($\chi^2_{df=9} = 45.265$, p < 0.001) and duration ($\chi^2_{df=9} = 37.823$, p < 0.001). In both cases, all intervals but the first after white noise offset showed less huddling than during the white noise (all p's < 0.03) (Fig. 9B-C). Sniffing the floor increased after white noise offset (frequency: $F_{2,9} = 3.243$, p = 0.002; duration: $F_{2,9} = 2.675$, p = 0.008). Notably, this behavior was more frequent and lasted longer between 50 and 150 s following white noise offset (all p's < 0.05) (Fig. 9D). Finally, the time spent in the open area increased after the offset ($F_{2,9} = 2.798$, p = 0.006). This increase became significant from 350 to 450 s after the offset (all p's < 0.05) (Fig. 9E).

3.4.3. Co-occurrence analysis (Fig. 10)

Following exposure to white noise, AAV-ERα, AAV-ERβ and AAV-luc in the CeA appeared in distinct clusters. AAV-luc was associated with sexual, prosocial and non-social behaviors. AAV-ERα was found in the same cluster as exploratory behaviors, while AAV-ERβ was associated with anti-social behaviors and risk assessment (Fig. 10A).

AAV-luc in the VMN was associated to sexual behaviors and risk assessment. AAV-ERα appeared linked to rejection and resting alone.
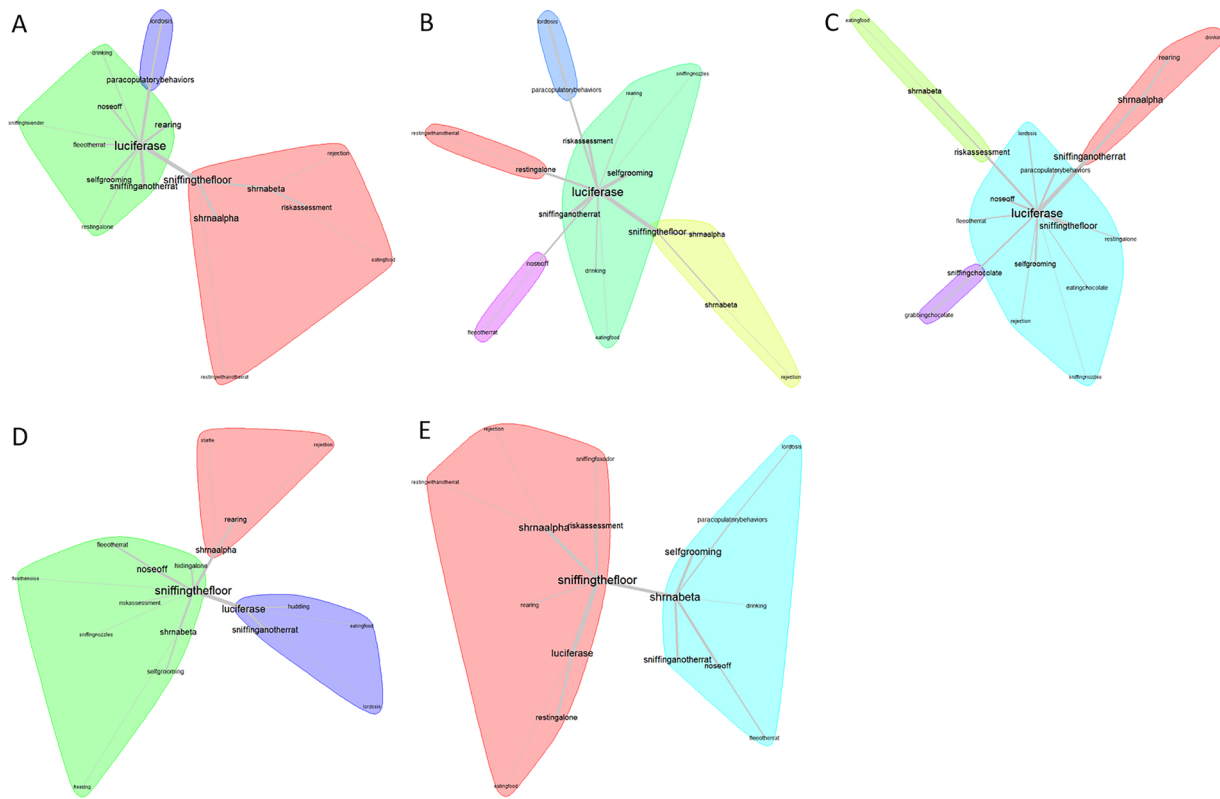
Fig. 8. Co-occurrence analysis showing main behavioral associations typical of each of the treatments in the VMN (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ), during exposure to (A) lavender odor, (B) music, (C) chocolate, (D) white noise and (E) fox odor (TMT). Clusters of behavioral association are represented in halos of different colors. 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.
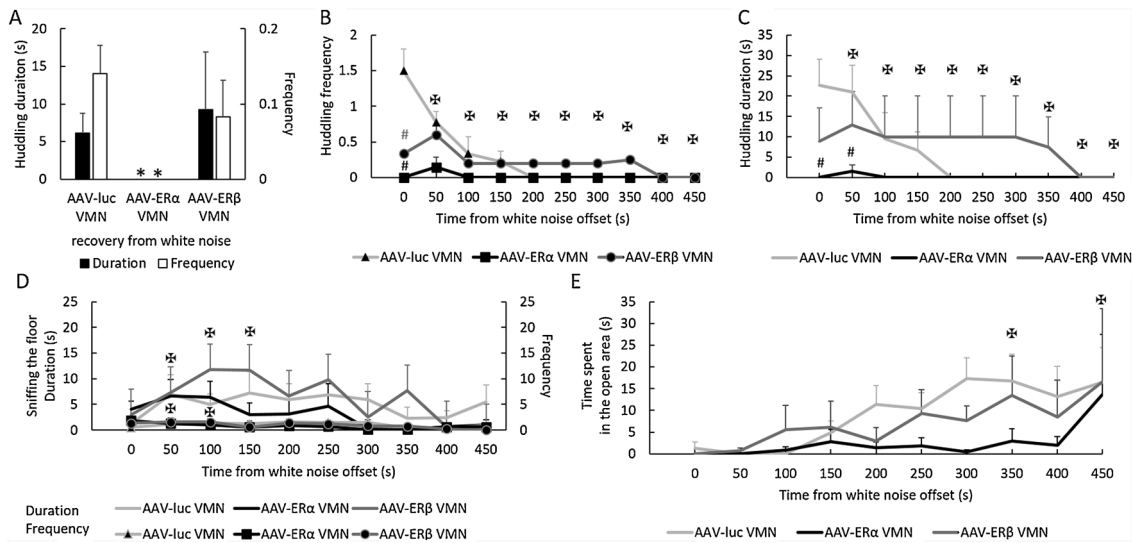
Fig. 9. (A) Frequency and duration of huddling during the 450 s following white noise offset in females infused with shRNA directed against the ERα or the ERβ in the VMN; *, different from AAV-luc. Huddling frequency (B), Huddling duration (C), Frequency and duration of sniffing the floor (D), Time spent in the open area (E), from the last 50-seconds of white noise exposure to 450 s after the offset of white noise, in females infused with shRNA directed against the ERα or the ERβ in the VMN. Each point represents behavior during the 50 s preceding it. Thus, 0 shows the behavior between -50 and 0 s after the end of white noise. Data are mean + SEM. ✠, different from the last interval of white noise exposure, all treatments collapsed; #, different from AAV-luc at the same time interval, the color of the # matches the color of the treatment exhibiting the difference.
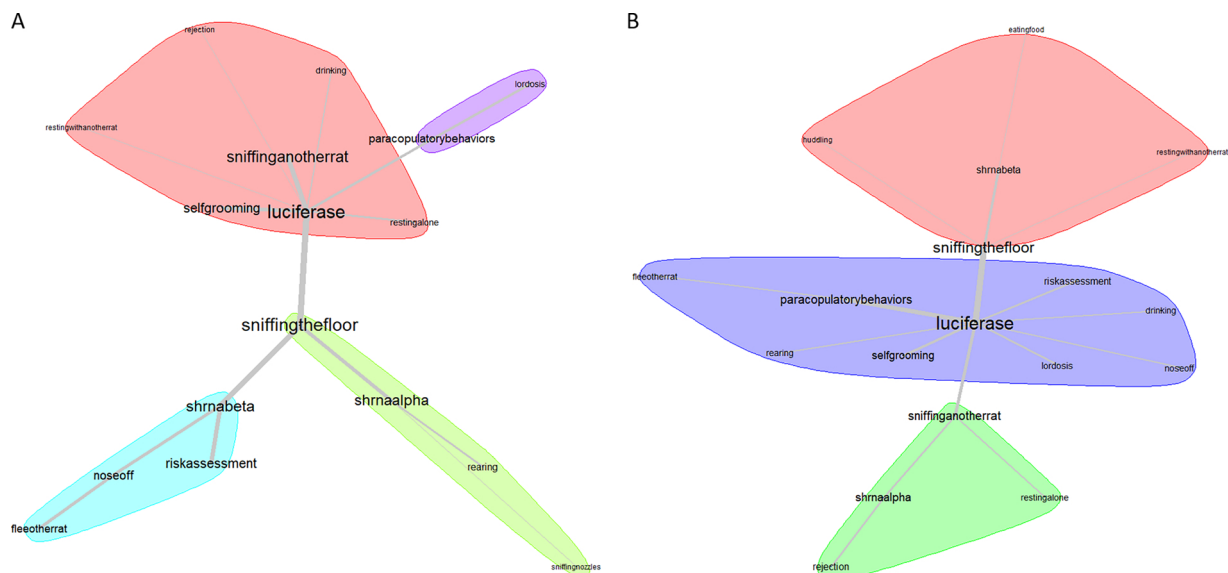
Fig. 10. Co-occurrence analysis showing main behavioral associations typical of each of the treatments (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ) during the 450 s following the exposure to white noise, (A) CeA and (B) VMN. Clusters of behavioral association are represented in halos of different colors. 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.

AAV-ERβ formed a distinct cluster with various behaviors: sniffing the floor, eating food, huddling and resting with another rat (Fig. 10B).

4. Discussion

4.1. Different emotional challenges elicit different behavioral patterns

The behavioral modifications induced by the different stimuli indicate that different emotional states were elicited in the female rats. Lavender increased exploration of the open area and stimulated olfactory investigation of males. Music reduced locomotory activity in the burrow and generally decreased olfactory exploration, as well as risk assessment. Chocolate was mainly characterized by chocolate-related behaviors and decreased the presence in the burrow. White noise exposure was strongly aversive to the rats: It increased behavioral indicators of fear, and also heightened the rat's arousal (e.g. locomotory activity). Fox odor increased the presence in the burrow and reduced social interactions. The effect of music is difficult to interpret. Considering the decrease in exploratory, sexual, anti-social and prosocial behavior, the most cautious conclusion is that music lowered rats' arousal.

The present results overall confirmed our predictions on the effect of positive and aversive stimuli on rat's behavior. These stimuli were able to elicit different levels of arousal and to modify classical indices of fear and anxiety, thus they are relevant for the investigation of the ERs role in safe vs. threatening contexts. In addition, we observed the first 7.5 min following the end of white noise. We expected that disrupting estrogen actions in the VMN or the CeA would influence the structure of behavioral recovery from white noise, and notably of behaviors specific to this stimulus. The post-white noise interval analyzed here showed that white noise specific behavior "huddling", and open area exploration returned to or approached baseline levels. This observation suggests that recovery from even a strongly aversive stimulus is rather quick. Therefore, the 50 min interval applied between the stimuli should be sufficient to avoid overlapping effects. Interestingly, the co-occurrence analyses of the post-white noise period confirmed the association between white noise and risk assessment in the AAV-ERβ group.

4.2. Estrogens receptors in the CeA regulate arousal and anxiety levels

Knockdown of the ERα in the CeA did not produce any observable effect. This was not unexpected, considering the few ERα receptors present in that area [20,28]. To the contrary, reduced expression of the ERβ in this structure enhanced risk assessment duration. In addition, in the co-occurrence analysis AAV-ERβ was associated with risk assessment display during exposure to lavender odor and music, and also in the minutes following white noise offset. In many of the standard tests for fear and anxiety, a similar behavior pattern is considered an exquisite indicator of the subject's level of anxiety [62–64]. Thus, the females with few ERβ receptors in the CeA showed enhanced anxiety. The reduced eating frequency is compatible with elevated anxiety levels. These observations clearly suggest that stimulation of this receptor at this site has anxiolytic actions. In the co-occurrence analysis, only during exposure to the strongly aversive white noise, AAV-ERα and AAV-ERβ appeared in the same cluster. A possible explanation is that this fear-inducing stimulus masked the anxiolytic effects of ERβ, which were more apparent during less aversive stimuli. We suggest that at least some of the anxiolytic actions of systemically administered ERβ agonists are localized to the CeA. In addition, AAV-ERβ increased sniffing the floor during all emotion-inducing stimuli. In the co-occurrence analysis, at each stimulus AAV-ERβ was associated with environmental exploration (sniffing floor and nozzles). It was also associated with chocolate investigation. All these behaviors are characteristic of increased arousal as operationally defined by Pfaff et al. [65].

A different question is whether estrogens, acting on the ERβ in the CeA, participates in the physiological regulation of fear and anxiety responses. There is little evidence for enhanced blood concentration of estrogens in stress- or fear-inducing contexts. In fact, foot shock or chronic mild stress have been reported to either leave blood estrogen concentrations unchanged [66] or to produce a small decrease [67,68]. Thus if estrogens would modulate the acute effects of stressors, it would be necessary to assume local synthesis of the steroid. Neurons in the amygdala express aromatase [69–71], making it possible to propose that estrogens indeed may be locally synthesized. There is actually some evidence showing that stressful events (foot shock) enhance the concentration of estradiol in the amygdala of female rats, without any concomitant change in plasma testosterone or estradiol [72]. These observations suggest enhanced local estrogen synthesis in the amygdala

in response to stress. Furthermore, since the availability of the substrate for aromatase, testosterone, does not increase [72], *de novo* steroid synthesis must be required. Unfortunately, none of the studies mentioned above distinguished between the different amygdaloid nuclei, but it may be assumed that also the CeA expresses aromatase, and that the stress-induced increase in aromatase expression and estrogen concentration also occur within this structure. If these speculations are correct, then activation of the ERβ in the CeA would attenuate the response to fear-inducing stimuli, and reduced expression of this receptor would enhance these responses, exactly as occurred in the present study. It must also be mentioned that many rapid actions of the ERβ have been described [73,74], making it possible for local synthesis to have almost immediate behavioral effects.

In this context it may be interesting to note that rats in proestrus and estrus show reduced anxiety on the elevated plus maze [75] and in the Vogel conflict procedure [76] as well as in the light-dark, social interaction and defensive burying tests [77]. A similar variation during the estrus cycle has been reported in mice [78]. However, ERβ knockout mice do not show this variation [79]. It appears, then, that the ERβ mediates the estrus cycle-associated variations in response to threatening situations, at least in mice. The specific role of the ERβ in the central amygdala has not been evaluated, but it is known that local injections of estradiol into the amygdala have anxiolytic effects [80]. Site-specific knockdown of the ERβ in cycling females could provide the data necessary for determining the role of the CeA in the variations in anxiety responses during the estrus cycle.

Finally, silencing either the ERα or the ERβ in the CeA had no influence on behavioral recovery after white noise exposure. Nevertheless, in the co-occurrence analysis, AAV-ERα, AAV-ERβ and AAV-luc appeared in distinct clusters. AAV-ERα was associated with exploratory behaviors, while AAV-ERβ appeared together with antisocial behaviors and risk assessment. Interestingly, the AAV-luc group was associated with sexual behaviors and resting. It is difficult to give a meaning to this observation. Perhaps ERs are differentially involved in responses to an aversive stimulus and recovery from these responses after the end of the stimulus.

4.3. Estrogens receptors in the VMN regulate sexual behaviors and possibly fear-related behaviors

The reduction in the number of ERα in the VMN was characterized by a diminution in sexual behaviors. The females were less likely to display lordosis. This is consistent with previous findings [7,8]. In addition, AAV-ERα was regularly associated with rejection in the co-occurrence analyses. The fact that lordosis was not entirely suppressed despite the strong reduction observed in the number of ERα (94%) could be due to a slightly too dorsal injection of the AAV in the VMN. Indeed, lordosis is mediated specifically by ERs in the ventro-lateral area of the VMN [81]. In the present study, we followed the usual procedure by counting the number of receptors in the entire VMN [7]. However, it appeared that about half of our rats infused with the shRNA directed against ERα in the VMN had the infusion cannulae tips located in the dorsal part of the nucleus. This could account for the fact that some females in the AAV-ERα group displayed lordosis.

Silencing of the ERα in the VMN seems to have anxiolytic properties. First, the behavior huddling during white noise exposure was suppressed by AAV-ERα. Rats seek social interaction in aversive situations to lower manifestations of fear, a phenomenon called social buffering [82]. Anxiolytic treatment has been found to decreased the need for social buffering [82,83]. Therefore, the decrease in huddling, but not hiding alone during aversive white noise, could be interpreted as an anxiolytic effect. After the offset of white noise, females treated with AAV-ERα huddled for a shorter time than the controls, while females treated with AAV-ERβ were not different. This suggests that silencing of the ERα is responsible for this anxiolytic action. Second, the frequent association of AAV-ERα with rearing, a novelty-induced behavior [84],

could also be interpreted as decreased anxiety [85,86]. If silencing the ERα in the VMN leads to reduced manifestation of anxiety-related behaviors in an aversive context, it can be concluded that this receptor is anxiogenic in such contexts. This is exactly what was proposed some years ago [87].

5. Conclusion

The main findings of this experiment were that the ERβ in the CeA is anxiolytic in several emotion-inducing contexts. To the contrary, in the VMN ERα appears to be anxiogenic in aversive contexts, while silencing ERβ had no effect.

We have previously argued that a seminatural environment has an external validity far superior to that of standard test procedures [43,88]. Consequently, we dare to propose that the effects observed here are manifestations of the importance of the ERs in rats' natural response to emotion-inducing stimuli. Finally, present data points to the CeA as a structure with an essential role in estrogens' emotion-modulating actions. Whether these observations are relevant or not for understanding the sexual dimorphisms in the prevalence of some psychiatric disorders in the human is impossible to determine at present.

References

[1] O. Le Moëne, A. Ågmo, Behavioral responses to emotional challenges in female rats living in a seminatural environment: the role of estrogen receptors, Horm. Behav. 106 (2018) 162–177, https://doi.org/10.1016/j.yhbeh.2018.10.013.

[2] S. Ogawa, V. Eng, J. Taylor, D.B. Lubahn, K.S. Korach, D.W. Pfaff, N. Carolina, Roles of estrogen receptor-alpha gene expression in reproduction-related behaviors in female mice, Endocrinology 139 (1998) 5070–5081.

[3] S. Ogawa, T.F. Washburn, J. Taylor, D.B. Lubahn, K.S. Korach, D.W. Pfaff, Modifications of testosterone-dependent behaviors by estrogen receptor- α gene disruption in male mice, Endocrinology 139 (1998) 5058–5069, https://doi.org/10.1210/endo.139.12.6358.

[4] S. Ogawa, J. Chan, A.E. Chester, J.-Å. Gustafsson, K.S. Korach, D.W. Pfaff, Survival of reproductive behaviors in estrogen receptor beta gene-deficient (beta ERKO) male and female mice, Proc. Natl. Acad. Sci. 96 (1999) 12887–12892, https://doi.org/10.1073/pnas.96.22.12887.

[5] S. Musatov, W. Chen, D.W. Pfaff, M.G. Kaplitt, S. Ogawa, RNAi-mediated silencing of estrogen receptor α in the ventromedial nucleus of hypothalamus abolishes female sexual behaviors, Proc. Natl. Acad. Sci. 103 (2006) 10456–10460, https://doi.org/10.1073/pnas.0603045103.

[6] E.F. Rissman, S.R. Wersinger, J.A. Taylor, D.B. Lubahn, Estrogen receptor function as revealed by knockout studies: neuroendocrine and behavioral aspects, Horm. Behav. 31 (1997) 232–243, https://doi.org/10.1006/hbeh.1997.1390.

[7] T. Spiteri, S. Musatov, S. Ogawa, A. Ribeiro, D.W. Pfaff, A. Ågmo, Estrogen-induced sexual incentive motivation, proceptivity and receptivity depend on a functional estrogen receptor α in the ventromedial nucleus of the hypothalamus but not in the amygdala, Neuroendocrinology 91 (2010) 142–154, https://doi.org/10.1159/000255766.

[8] C.A. Mazzucco, H.A. Walker, J.L. Pawluski, S.E. Lieblich, L.A.M. Galea, ERα, but not ERβ, mediates the expression of sexual behavior in the female rat, Behav. Brain Res. 191 (2008) 111–117, https://doi.org/10.1016/j.bbr.2008.03.016.

[9] A.A. Walf, I. Ciriza, L.M. Garcia-Segura, C.A. Frye, Antisense oligodeoxynucleotides for estrogen receptor-β and α attenuate estradiol's modulation of affective and sexual behavior, respectively, Neuropsychopharmacology 33 (2008) 431–440, https://doi.org/10.1038/sj.npp.1301416.

[10] G.G. Nomikos, C. Spyraki, Influence of oestrogen on spontaneous and diazepam-induced exploration of rats in an elevated plus maze, Neuropharmacology 27

(1988) 691–696.

[11] A.A. Walf, C.A. Frye, Effects of two estradiol regimens on anxiety and depressive behaviors and trophic effects in peripheral tissues in a rodent model, Gend. Med. 6 (2009) 300–311, https://doi.org/10.1016/j.genm.2009.04.004.

[12] A.A. Walf, C.A. Frye, ERβ-selective estrogen receptor modulators produce anti-anxiety behavior when administered systemically to ovariectomized rats, Neuropsychopharmacology 30 (2005) 1598–1609, https://doi.org/10.1038/sj.npp.1300713.

[13] B.D. Imwalle, J.-A. Gustafsson, E.F. Rissman, Lack of functional estrogen receptor β influences anxiety behavior and serotonin content in female mice, Physiol. Behav. 84 (2005) 157–163, https://doi.org/10.1016/j.physbeh.2004.11.002.

[14] T.D. Lund, T. Rovis, W.C.J. Chung, R.J. Handa, Novel actions of estrogen receptor-β on anxiety-related behaviors, Endocrinology 146 (2005) 797–807, https://doi.org/10.1210/en.2004-1158.

[15] K. Tomihara, T. Soga, M. Nomura, K.S. Korach, J.Å. Gustafsson, D.W. Pfaff, S. Ogawa, Effect of ER-β gene disruption on estrogenic regulation of anxiety in female mice, Physiol. Behav. 96 (2009) 300–306, https://doi.org/10.1016/j.physbeh.2008.10.014.

[16] D.J. Toufexis, K.M. Myers, M.E. Bowser, M. Davis, Estrogen disrupts the inhibition of fear in female rats, possibly through the antagonistic effects of estrogen receptor α (ERα) and ERβ, J. Neurosci. 27 (2007) 9729–9735, https://doi.org/10.1523/JNEUROSCI.2529-07.2007.

[17] M. Furuta, T. Numakawa, S. Chiba, M. Ninomiya, Y. Kajiyama, N. Adachi, T. Akema, H. Kunugi, Estrogen, predominantly via estrogen receptor α, attenuates postpartum-induced anxiety- and depression-like behaviors in female rats, Neuroendocrinology 154 (2013) 3807–3816, https://doi.org/10.1210/en.2012-2136.

[18] M.A. Morgan, D.W. Pfaff, Effects of estrogen on activity and fear-related behaviors in mice, Horm. Behav. 40 (2001) 472–482, https://doi.org/10.1006/hbeh.2001.1716.

[19] T. Spiteri, S. Musatov, S. Ogawa, A. Ribeiro, D.W. Pfaff, A. Ågmo, The role of the estrogen receptor α in the medial amygdala and ventromedial nucleus of the hypothalamus in social recognition, anxiety and aggression, Behav. Brain Res. 210 (2010) 211–220, https://doi.org/10.1016/j.bbr.2010.02.033.

[20] P.J. Shughrue, M.V. Lane, I. Merchenthaler, Comparative distribution of estrogen receptor-α and -β mRNA in the rat central nervous system, J. Comp. Neurol. 388 (1997) 507–525.

[21] S.W. Mitra, E. Hoskin, J. Yudkovitz, L. Pear, H.A. Wilkinson, S. Hayashi, D.W. Pfaff, S. Ogawa, S.P. Rohrer, J.M. Schaeffer, B.S. McEwen, S.E. Alves, Immunolocalization of estrogen receptor β in the mouse brain: comparison with estrogen receptor α, Endocrinology 144 (2003) 2055–2067, https://doi.org/10.1210/en.2002-221069.

[22] C. Orikasa, Y. Sakuma, Sex and region-specific regulation of oestrogen receptor β in the rat hypothalamus, J. Neuroendocrinol. 16 (2004) 964–969, https://doi.org/10.1111/j.1365-2826.2004.01254.x.

[23] P. Sah, E.S.L. Faber, M. Lopez De Armentia, J. Power, The amygdaloid complex: anatomy and physiology, Physiol. Rev. 83 (2003) 803–834, https://doi.org/10.1152/physrev.00002.2003.

[24] K.L. Kopchia, H.J. Altman, R.L. Commissaris, Effects of lesions of the central nucleus of the amygdala on anxiety-like behaviors in the rat, Pharmacol. Biochem. Behav. 43 (1992) 453–461, https://doi.org/10.1016/0091-3057(92)90176-G.

[25] K. Shibata, Y. Kataoka, K. Yamashita, S. Ueki, An important role of the central amygdaloid nucleus and mammillary body in the mediation of conflict behavior in rats, Brain Res. 372 (1986) 159–162, https://doi.org/10.1016/0006-8993(86)91470-8.

[26] T. Paretkar, E. Dimitrov, The central amygdala corticotropin-releasing hormone (CRH) neurons modulation of anxiety-like behavior and hippocampus-dependent memory in mice, Neuroscience 390 (2018) 187–197, https://doi.org/10.1016/j.neuroscience.2018.08.019.

[27] A. Korosi, T.Z. Baram, The central corticotropin releasing factor system during development and adulthood, Eur. J. Pharmacol. 583 (2008) 204–214, https://doi.org/10.1016/j.ejphar.2007.11.066.

[28] M. Österlund, G.G.J.M. Kuiper, J.-A. Gustafsson, Y.L. Hurd, Differential distribution and regulation of estrogen receptor-α and-β mRNA within the female rat brain, Mol. Brain Res. 54 (1998) 175–180.

[29] P.J. Shughrue, I. Merchenthaler, Distribution of estrogen receptor β immunoreactivity in the rat central nervous system, J. Comp. Neurol. 436 (2001) 64–81, https://doi.org/10.1002/cne.1054.

[30] M.J. Weiser, C.D. Foradori, R.J. Handa, Estrogen receptor beta activation prevents glucocorticoid receptor-dependent effects of the central nucleus of the amygdala on behavior and neuroendocrine function, Brain Res. 1336 (2010) 78–88, https://doi.org/10.1016/j.brainres.2010.03.098.

[31] C.D. Foradori, T.D. Lund, A.H. Nagahara, J.I. Koenig, R.J. Handa, Corticotropin-releasing hormone heterogeneous nuclear RNA (hnRNA) and immunoreactivity are induced in extrahypothalamic brain sites by kainic-acid-induced seizures and are modulated by estrogen, Brain Res. 1164 (2007) 44–54, https://doi.org/10.1016/j.brainres.2007.05.064.

[32] C. Lampert, D.M. Arcego, D.P. Laureano, L.A. Diehl, I.F. da Costa Lima, R. Krolow, L.F. Pettenuzzo, C. Dalmaz, D. Vendite, Effect of chronic administration of tamoxifen and/or estradiol on feeding behavior, palatable food and metabolic parameters in ovariectomized rats, Physiol. Behav. 119 (2013) 17–24, https://doi.org/10.1016/j.physbeh.2013.05.026.

[33] D. Shaw, J.M. Annett, B. Doherty, J.C. Leslie, Anxiolytic effects of lavender oil inhalation on open-field behaviour in rats, Phytomedicine 14 (2007) 613–620, https://doi.org/10.1016/j.phymed.2007.03.007.

[34] M.L. Reynaert, J. Marrocco, J. Mairesse, L. Lionetto, M. Simmaco, L. Deruyter, D. Allorge, A. Moles, A. Pittaluga, S. Maccari, S. Morley-Fletcher, G. Van Camp,

[35] F. Nicoletti, Hedonic sensitivity to natural rewards is affected by prenatal stress in a sex-dependent manner, Addict. Biol. 21 (2016) 1072–1085, https://doi.org/10.1111/adb.12270.

[35] P. Weyers, W. Janke, M. Macht, H.G. Weijers, Social and non-social open field behaviour of rats under light and noise stimulation, Behav. Processes 31 (1994) 257–267, https://doi.org/10.1016/0376-6357(94)90011-6.

[36] T. Endres, R. Apfelbach, M. Fendt, Behavioral changes induced in rats by exposure to trimethylthiazoline, a component of fox odor, Behav. Neurosci. 119 (2005) 1004–1010, https://doi.org/10.1037/0735-7044.119.4.1004.

[37] B. Escribano, I. Quero, M. Feijóo, I. Tasset, P. Montilla, I. Túnez, Role of noise and music as anxiety modulators: relationship with ovarian hormones in the rat, Appl. Anim. Behav. Sci. 152 (2014) 73–82, https://doi.org/10.1016/j.applanim.2013.12.006.

[38] P. Pinares-Garcia, M. Stratikopoulos, A. Zagato, H. Loke, J. Lee, Sex: a significant risk factor for neurodevelopmental and neurodegenerative disorders, Brain Sci. 8 (2018) 154, https://doi.org/10.3390/brainsci8080154.

[39] M.B. Solomon, J.P. Herman, Sex differences in psychopathology: of gonads, adrenals and mental illness, Physiol. Behav. 97 (2009) 250–258, https://doi.org/10.1016/j.physbeh.2009.02.033.

[40] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, San Diego, CA, 1998.

[41] M. Nakata, K. Sano, S. Musatov, N. Yamaguchi, T. Sakamoto, S. Ogawa, Effects of prepubertal or adult site-specific knockdown of estrogen receptor β in the medial preoptic area and medial amygdala on social behaviors in male mice, ENeuro 3 (2016) 155–170, https://doi.org/10.1523/ENEURO.0155-15.2016.

[42] O. Le Moëne, A. Ågmo, Responses to positive and aversive stimuli in estrous female rats housed in a seminatural environment: Effets of yohimbine and chlordiazepoxide, Pharmacol. Biochem. Behav. 179 (2019) 43–54, https://doi.org/10.1016/j.pbb.2019.02.001.

[43] X. Chu, A. Ågmo, Sociosexual behaviours in cycling, intact female rats (Rattus norvegicus) housed in a seminatural environment, Behaviour 151 (2014) 1143–1184, https://doi.org/10.1163/1568539X-00003177.

[44] X. Chu, A. Ågmo, Sociosexual behaviors of male rats (Rattus norvegicus) in a seminatural environment, J. Comp. Psychol. 129 (2015) 132–144, https://doi.org/10.1037/a0038722.

[45] B.F. Bradley, S.L. Brown, S. Chu, R.W. Lea, Effects of orally administered lavender essential oil on responses to anxiety-provoking film clips, Hum. Psychopharmacol. 24 (2009) 319–330, https://doi.org/10.1002/hup.1016.

[46] J. Cruz, D. Lima, D. Dal Magro, J. Cruz, Anxiolytic effect of Mozart music over short and long photoperiods as part of environmental enrichment in captive Rattus norvegicus (Rodentia: Muridae), Scand. J. Lab. Anim. Sci. 41 (2015) 1–7.

[47] A.Y.R. Kühlmann, A. de Rooij, M.G.M. Huninк, C.I. De Zeeuw, J. Jeekel, Music affects rodents: a systematic review of experimental research, Front. Behav. Neurosci. 12 (2018) 1–19, https://doi.org/10.3389/fnbeh.2018.00301.

[48] I. La Mela, E.C. Latagliata, E. Patrono, S. Puglisi-Allegra, R. Ventura, Olfactory priming reinstates extinguished chocolate-induced conditioned place preference, Appetite 54 (2010) 237–240, https://doi.org/10.1016/j.appet.2009.12.008.

[49] S. Koba, R. Inoue, T. Watanabe, Role played by periaqueductal gray neurons in parasympathetically mediated fear bradycardia in conscious rats, Physiol. Rep. 4 (2016) 1–13, https://doi.org/10.14814/phy2.12831.

[50] M. Fendt, T. Endres, C.A. Lowry, R. Apfelbach, I.S. McGregor, TMT-induced autonomic and behavioral changes and the neural basis of its processing, Neurosci. Biobehav. Rev. 29 (2005) 1145–1156, https://doi.org/10.1016/j.neubiorev.2005.04.018.

[51] D. Homiack, E. O'Cinneide, S. Hajmurad, B. Barrileaux, M. Stanley, M.R. Kreutz, L.A. Schrader, Predator odor evokes sex-independent stress responses in male and female Wistar rats and reduces phosphorylation of cyclic-adenosine monophosphate response element binding protein in the male, but not the female hippocampus, Hippocampus 27 (2017) 1016–1029, https://doi.org/10.1002/hipo.22749.

[52] G. Buchbauer, L. Jirovetz, W. Jäger, Aromatherapy: evidence for sedative effects of the essential oil of lavender after inhalation, Zeitschrift Für Naturforsch. C. 46 (1991) 1067–1072.

[53] K.J. Wallace, J.B. Rosen, Predator odor as an unconditioned fear stimulus in rats: elicitation of freezing by trimethylthiazoline, a component of fox feces, Behav. Neurosci. 114 (2000) 912–922, https://doi.org/10.1037/0735-7044.114.5.912.

[54] M.A. Snyder, T. Smejkalova, P.M. Forlano, C.S. Woolley, Multiple ERβ antisera label in ERβ knockout and null mouse tissues, J. Neurosci. Methods 188 (2010) 226–234, https://doi.org/10.1016/J.JNEUMETH.2010.02.012.

[55] K. Charitidi, I. Meltser, B. Canlon, Estradiol treatment and hormonal fluctuations during the estrous cycle modulate the expression of estrogen receptors in the auditory system and the prepulse inhibition of acoustic startle response, Endocrinology 153 (2012) 4412–4421, https://doi.org/10.1210/en.2012-1416.

[56] S.X. Simonian, A.E. Herbison, Differential expression of estrogen receptor α and β immunoreactivity by oxytocin neurons of rat paraventricular nucleus, J. Neuroendocrinol. 9 (1997) 803–806.

[57] P. Marchand, P. Ratinaud, L'analyse de similitude appliquée aux corpus textuels : les primaires socialistes pour l'élection présidentielle française (septembre-octobre 2011), Actes Des 11eme Journées Int. d'Analyse Stat. Des Données Textuelles, JADT 2012, Liège, 2012, pp. 687–698.

[58] M. Reinert, Une méthode de classification descendante hiérarchique: Application à l'analyse lexicale par contexte, Les Cah. l'analyse Données 8 (1983) 187–198.

[59] M. Reinert, Alceste une méthodologie d'analyse des données textuelles et une application: Aurelia De Gerard De Nerval, Bull. Méthodologie Sociol. 26 (1990) 24–54, https://doi.org/10.1177/075910639002600103.

[60] T. Spiteri, S. Ogawa, S. Musatov, D.W. Pfaff, A. Ågmo, The role of the estrogen

receptor α in the medial preoptic area in sexual incentive motivation, proceptivity and receptivity, anxiety, and wheel running in female rats, Behav. Brain Res. 230 (2012) 11–20, https://doi.org/10.1016/j.bbr.2012.01.048.

[61] E.M.S. Snoeren, E. Antonio-Cabrera, T. Spiteri, S. Musatov, S. Ogawa, D.W. Pfaff, A. Ågmo, Role of oestrogen α receptors in sociosexual behaviour in female rats housed in a seminatural environment, J. Neuroendocrinol. 27 (2015) 803–818, https://doi.org/10.1111/jne.12321.

[62] D.C. Blanchard, R.J. Blanchard, P. Tom, R.J. Rodgers, Psychopharmacology Diazepam changes risk assessment in an anxiety / defense test battery, Psychopharmacology (Berl) 101 (1990) 511–518.

[63] D.C. Blanchard, Risk assessment: At the interface of cognition and emotion, Curr. Opin. Behav. Sci. 24 (2018) 69–74, https://doi.org/10.1016/j.cobeha.2018.03.006.

[64] A.P. Carobrez, L.J. Bertoglio, Ethological and temporal analyses of anxiety-like behavior: the elevated plus-maze model 20 years on, Neurosci. Biobehav. Rev. 29 (2005) 1193–1205, https://doi.org/10.1016/j.neubiorev.2005.04.017.

[65] D.W. Pfaff, Brain Arousal and Information Theory: Neural and Genetic Mechanisms, Harvard University Press, 2006.

[66] L. Guo, Y.X. Chen, Y.T. Hu, X.Y. Wu, Y. He, J.L. Wu, M.L. Huang, M. Mason, A.M. Bao, Sex hormones affect acute and chronic stress responses in sexually dimorphic patterns: Consequences for depression models, Psychoneuroendocrinology 95 (2018) 34–42, https://doi.org/10.1016/j.psyneuen.2018.05.016.

[67] X. Fu, H. Chen, N. Zhang, M. Ding, Y. Qiu, X. Pan, Y. Fang, Y. Lin, Q. Zheng, W. Wang, Effects of chronic unpredictable mild stress on ovarian reserve in female rats: feasibility analysis of a rat model of premature ovarian failure, Mol. Med. Rep. 18 (2018) 532–540, https://doi.org/10.3892/mmr.2018.8989.

[68] J. Lu, X.Y. Wu, Q. Bin Zhu, J. Li, L.G. Shi, J.L. Wu, Q.J. Zhang, M.L. Huang, A.M. Bao, Sex differences in the stress response in SD rats, Behav. Brain Res. 284 (2015) 231–237, https://doi.org/10.1016/j.bbr.2015.02.009.

[69] R.L. Jakab, T.L. Horvath, C. Leranth, N. Harada, F. Naftolin, Aromatase immunoreactivity in the rat brain: gonadectomy-sensitive hypothalamic neurons and an unresponsive "limbic ring" of the lateral septum-bed nucleus-amygdala complex, J. Steroid Biochem. Mol. Biol. 44 (1993) 481–498, https://doi.org/10.1016/0960-0760(93)90253-S.

[70] J. Li, P.J. Oberly, S.M. Poloyac, R.B. Gibbs, A microsomal based method to detect aromatase activity in different brain regions of the rat using ultra performance liquid chromatography–mass spectrometry, J. Steroid Biochem. Mol. Biol. 163 (2016) 113–120, https://doi.org/10.1016/j.jsbmb.2016.04.013.

[71] F. Naftolin, T.L. Horvath, R.L. Jakab, C. Leranth, N. Harada, J. Balthazart, Aromatase immunoreactivity in axon terminals of the vertebrate brain, Neuroendocrinology 63 (1996) 149–155, https://doi.org/10.1159/000126951.

[72] V.A. Sashkov, N.B. Selverova, E.D. Morenkov, I.V. Ermakova, Level of neuroactive steroids in the brain and sex-related peculiarities of formation and extinction of conditioned reflex in rats, J. Evol. Biochem. Physiol. 46 (2010) 366–373, https://doi.org/10.1134/S0022093010040058.

[73] K.G. Vargas, J. Milic, A. Zaciragic, L. xin Wen, L. Jaspers, J. Nano, K. Dhana, W.M. Bramer, B. Kraja, E. van Beeck, M.A. Ikram, T. Muka, O.H. Franco, The functions of estrogen receptor beta in the female brain: a systematic review,

Maturitas 93 (2016) 41–57, https://doi.org/10.1016/j.maturitas.2016.05.014.

[74] J.M. Lymer, P.A.S. Sheppard, T. Kuun, A. Blackman, N. Jani, S. Mahbub, E. Choleris, Estrogens and their receptors in the medial amygdala rapidly facilitate social recognition in female mice, Psychoneuroendocrinology 89 (2018) 30–38, https://doi.org/10.1016/J.PSYNEUEN.2017.12.021.

[75] F.K. Marcondes, K.J. Miguel, L.L. Melo, R.C. Spadari-Bratfisch, Estrous cycle influences the response of female rats in the elevated plus-maze test, Physiol. Behav. 74 (2001) 435–440, https://doi.org/10.1016/S0031-9384(01)00593-5.

[76] M. Molina-Hernández, C.M. Contreras, P. Téllez-Alcántara, Diazepam increases the number of punished responses in a conflict-operant paradigm during late proestrus and estrus in the Wistar rat, Neuropsychobiology 43 (2001) 29–33.

[77] C.A. Frye, S.M. Petralia, M.E. Rhodes, Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3α,5α-THP, Pharmacol. Biochem. Behav. 67 (2000) 587–596, https://doi.org/10.1016/S0091-3057(00)00392-0.

[78] P. Palanza, L. Gioiosa, S. Parmigiani, Social stress in mice: gender differences and effects of estrous cycle and social dominance, Physiol. Behav. 73 (2001) 411–420, https://doi.org/10.1016/S0031-9384(01)00494-2.

[79] A.A. Walf, C. Koonce, K. Manley, C.A. Frye, Proestrous compared to diestrous wildtype, but not estrogen receptor beta knockout, mice have better performance in the spontaneous alternation and object recognition tasks and reduced anxiety-like behavior in the elevated plus and mirror maze, Behav. Brain Res. 196 (2009) 254–260, https://doi.org/10.1016/J.BBR.2008.09.016.

[80] C.A. Frye, A.A. Walf, Estrogen and/or progesterone administered systemically or to the amygdala can have anxiety-, fear-, and pain-reducing effects in ovariectomized rats, Behav. Neurosci. 118 (2004) 306–313.

[81] D.W. Pfaff, Y. Sakuma, Facilitation of the lordosis reflex of female rats from the ventromedial nucleus of the hypothalamus, J. Physiol. 288 (1979) 189–202.

[82] Y. Kiyokawa, M.B. Hennessy, Comparative studies of social buffering: a consideration of approaches, terminology, and pitfalls, Neurosci. Biobehav. Rev. 86 (2018) 131–141, https://doi.org/10.1016/j.neubiorev.2017.12.005.

[83] G.T. Taylor, Fear and affiliation in domesticated male rats, J. Comp. Physiol. Psychol. 95 (1981) 685–693, https://doi.org/10.1037/h0077817.

[84] J.L. Wolfe, Observations on alertness and exploratory behavior in the eastern chipmunk, Am. Midl. Nat. 81 (1969) 249–253.

[85] A.G. Nasello, C. Machado, J.F. Bastos, L.F. Felicio, Sudden darkness induces a high activity-low anxiety state in male and female rats, Physiol. Behav. 63 (1998) 451–454.

[86] I. Oloruntobi, O. Ajayi, I. Rufus, Anxiolytic, sedative and hypothermic effects of aqueous leaf extract of Vernonia amygdalina Del. (Asteraceae) in albino mice, Br. J. Pharm. Res. 4 (2014) 2210–2225, https://doi.org/10.9734/BJPR/2014/12529.

[87] M.A. Morgan, J. Schulkin, D.W. Pfaff, Estrogens and non-reproductive behaviors related to activity and fear, Neurosci. Biobehav. Rev. 28 (2004) 55–63, https://doi.org/10.1016/j.neubiorev.2003.11.017.

[88] X. Chu, A. Ågmo, Sociosexual interactions in rats: Are they relevant for understanding human sexual behavior? Int. J. Psychol. Res. 9 (2016) 76–95, https://doi.org/10.21500/20112084.2339.