Responses to positive and aversive stimuli in estrous female rats housed in a seminatural environment: Effects of yohimbine and chlordiazepoxide[☆],[☆☆]

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ABSTRACT

The behavioral effects of putative anxiolytic and anxiogenic drugs are usually evaluated in highly standardized tests. Here, we determined the effects of such drugs in rats housed in mixed sex groups in a seminatural environment. Sexually receptive female Wistar rats were treated with either the anxiolytic drug chlordiazepoxide (2 mg/kg), the anxiogenic drug yohimbine (1 mg/kg), or saline (1 ml/kg). Different emotional challenges eliciting purportedly positive affect (lavender odor, Mozart's music, chocolate flavored food) or negative affect (white noise, fox odor) were then introduced into the seminatural environment. A co-occurrence analysis revealed that music was rather aversive to the rats, as were white noise and fox odor. Lavender and chocolate exposure decreased classical indicators of fear. White noise suppressed sexual behaviors and caused avoidance of the open area. Yohimbine increased sexual receptivity during lavender exposure, decreased the latency to flee the white noise, and increased self-grooming regardless of the emotional challenge. Chlordiazepoxide was effective only during exposure to white noise, and increased the frequency of hiding alone. The modest effects of the drugs in the seminatural environment may be the result of social buffering and rats experiencing a high degree of controllability over their environment.

1. Introduction

Clinically efficient anxiolytic drugs have been reported to alter behavior in several species of non-human mammals, particularly in contexts producing fear and stress. Thousands or hundreds of studies offer detailed descriptions of drug effects in popular procedures like the elevated plus maze, the Vogel conflict procedure, the social interaction test, and the light-dark choice test, just to offer a few examples. With the exception of the social interaction test, all these procedures are based on observation of a single animal at a time. The most commonly used animal, the rat, is well known to be a gregarious, usually group-living rodent (e.g. Eckman et al., 1969; Latané, 1969; Latané and Glass, 1968; Robitaille and Bouvet, 1976; Telle, 1966; Weiss et al., 2018). The usual habitat consists of a burrow and a variable area around the burrow, the home range (Barnett, 1975; Calhoun, 1962; Harper and Rutherford, 2016). Even though the rat is a gregarious animal, it engages in a number of solitary activities, for example foraging and scavenging in unfamiliar territory. Occurrences of solitary exploration do not modify the belonging to a social group. Thus, the standard tests for anxiety have eliminated the main social and physical features of the

rat's natural environment. Since most studies of fear and anxiety are concerned with the actions of drugs, and with the potential of these drugs for altering anxiety responses in the human, absence of these basic environmental features is of no concern. It is rather the predictive validity of the test procedures that is of major importance. The many criteria proposed for evaluating predictive validity has been reviewed a number of times (e.g. Cryan and Sweeney, 2012; de Boer and Koolhaas, 2003; Ramos, 2008; Treit, 1985; Willner and Mitchell, 2002), and will not be mentioned here.

In addition to predictive validity, experimental procedures may or may not have external validity. This concept refers to the generalizability of observations from a specific procedure to other procedures and to contexts outside the laboratory. In the brunswikian tradition (Brunswik, 1955), an externally valid procedure should incorporate as many as possible of the features of the experimental subject's natural habitat (Petrinovich, 1980; Petrinovich, 1989). The limited external validity of most tests of anxiety makes it difficult to generalize potential consequences of reduced or heightened fear or anxiety for the rat's behavior outside of the specific procedure employed. This becomes of concern when speculations about the biological function (or adaptive

value) of anxiety or fear reactions are presented. Such speculations usually refer to general behavioral processes, operating in situations outside the specific testing procedure employed. Here, external validity of the test becomes essential.

We have previously employed a seminatural environment (based on the one used by McClintock and Adler, 1978), consisting of a complex burrow system and a large open area, for analyzing several aspects of social behavior in groups of male and female rats (e.g. Chu and Ågmo, 2014; Chu and Ågmo, 2015; Chu et al., 2015). The procedure can be considered externally valid, because it incorporates the basic physical and social elements of rats' natural habitat (see Chu and Ågmo, 2016 for an extensive discussion), and it allows the animals to express a substantial proportion of their behavioral repertoire. Recently, we have enriched the procedure by introducing a series of emotional challenges in the environment, both positive and negative (Le Moëne and Ågmo, 2018).

In the present study, we employed the enriched procedure in order to determine the effects of either an anxiolytic (chlordiazepoxide) or an anxiogenic compound (yohimbine). Both drugs have been shown to effectively reduce and enhance anxiety, respectively, in female rats (see Basso et al., 2011; Smith et al., 2013 and references therein). The purpose of the study was twofold. First, it would allow us to determine the potential effects of fear- or anxiety-altering compounds on different behavior patterns in a procedure with external validity. We predicted that chlordiazepoxide would reduce behaviors indicative of fear in the fear-inducing situations, while it would have no effect in pleasant situations. Yohimbine would enhance fear-related behaviors in all situations. Second, the present experiment would make it possible to determine whether enhanced or reduced anxiety would affect sexual interactions. There has been much controversy around the role of stress and anxiety for sexual behaviors. Most experimental data suggest that acute stress preceding sexual interaction facilitates female sexual behavior (Brotto et al., 1999; Williams et al., 1992), whereas stress during sexual interaction is inhibiting (Le Moëne and Ågmo, 2018). Here, we predicted that the anxiolytic effects of chlordiazepoxide should enhance sexual interaction in fear-inducing situations, whereas the anxiogenic actions of yohimbine should reduce sexual activity in all situations. These data should provide some useful information about the role of fear and anxiety for socio-sexual interactions in a test procedure with some degree of external validity.

2. Material and methods

2.1. Subjects

Fifty-six Wistar rats from Charles River WIGA (Sulzfeld, Germany) were included in this study, (32 females, 250 g and 24 males, 300 g upon arrival). All animals were housed in same-sex pairs in standard Macrolon® IV cages, with food (RM1, Special Diets Services, Witham, UK) and water available ad libitum. The room hosting the rats was maintained at a temperature of 21 ± 1 °C with a humidity of 55 ± 10%. The ambient sound level, produced by the ventilation system, was about 40 dB. The light cycle was set on a reversed 12:12 h cycle, lights being on between 23:00 and 11:00 h. The females were ovariectomized under isofluorane anesthesia 14 days before their introduction into the seminatural environment.

This experiment was approved by the Norwegian Food Safety Authority (authorization 7102) and is in accordance with the European Union council directive 2010/63/EU.

2.2. Apparatus

The seminatural environment has been extensively described in Chu and Ågmo (2014, 2015). It consists of an open area (120 ∗ 210 cm) connected to a burrow with several corridors and 4 nest boxes (Fig. 1). The burrow was maintained in complete darkness for the whole

duration of the experiment, but infrared lights (850 nm) allowed for video recording. The light in the open area was set to the same reversed 12:12 light cycle as previously mentioned. As measured on the ground level, the light intensity at night was 30 lx, and 180 lx during the day. Artificial dawns and dusks were created by 30-min transitions from dark to light, and inversely.

The entire experiment was recorded by two digital cameras fixed to the ceiling about 2 m above the floor of the open area and burrow, respectively. The Media Recorder 2.5 (Noldus, Wageningen, The Netherlands) was used for creating and storing the video files.

2.3. Hormones and drugs

Estrus was induced by subcutaneous (SC) injections of estradiol benzoate (EB) and progesterone (P) (both from Sigma Aldrich, St Louis, MO) dissolved in peanut oil (Den norske Eterfabrikk, Norway) in a dose of 18 μg/kg and 1 mg/rat, respectively. The injection volume was 1 ml/kg for EB and 0.2 ml/rat for P. P was administered 48 h after EB.

Chlordiazepoxide and yohimbine were obtained from La Roche, Basel, Switzerland and SIGMA, St Louis, MO, respectively. Both were dissolved in saline, and the doses were 2 mg/kg and 1 mg/kg body weight, respectively. The drugs were injected SC in a volume of 1 ml/kg.

The doses of EB and P employed here have been used successfully in several earlier studies. They produce close to maximal receptivity and high intensity of paracopulatory behaviors (see Spiteri and Ågmo, 2006). Yohimbine is known to produce anxiogenic responses in intact female rats in the dose employed here (Smith et al., 2013). That dose has only slight sedative effect (Ventura-Aquino and Fernández-Guasti, 2013). The chlordiazepoxide dose used here produces anxiolytic effects in female rats in several procedures (Bonuti and Morato, 2018; Van Haaren and Zarcone, 1994) while having no sedative effects (Hughes and Syme, 1972). It was considered important to use doses with clear anxiolytic and anxiogenic effects, but with no or slight effect on motor functions. Otherwise, potential behavioral effects would be difficult to interpret.

It could be argued that it is inappropriate to base the choice of doses on studies performed in standard procedures, and that we should have established a full dose-effect curve. This would certainly have been desirable, but the time investment required for behavioral observations in our procedure precluded such an approach. It may also be pointed out that the doses of chlordiazepoxide and yohimbine used here are active in different standard procedures, as already mentioned, making it reasonable to assume that they should be active also in the present procedure.

2.4. Emotion-inducing events

The emotion-inducing events used to induce positive and negative affect have already been extensively described in Le Moëne and Ågmo (2018). Briefly, the rats were exposed to 5 emotion-inducing events:

1. Lavender odor from 1.5 ml *Lavandula angustifolia* essential oil (AromaBio, Lyon, France) deposited on a cotton pad, was distributed through a 3 l/min air stream for 30 min (Olfactory Stimulus Package, Medical Associates, Georgia, Vt). The air stream entered the seminatural environment through two nozzles, one placed on the wall of the open area, and one on a wall in a tunnel. Lavender odor has been reported to have anxiolytic effects in rats (Shaw et al., 2007; Umezu et al., 2006) and it seems to induce a positive affective state in rats and humans (Bradley et al., 2009).
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) at 55–60 dB for 24 min and 18 s. A lesser-known effect of music is to modulate anxiety. Exposure to this sonata showed decreased indicators of
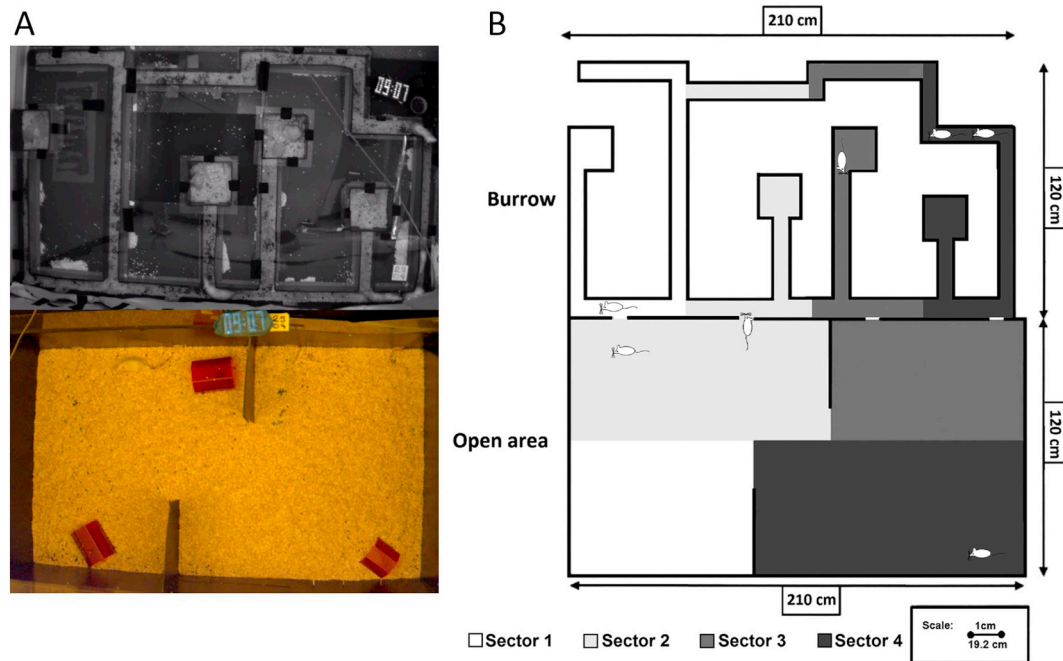
Fig. 1. A. Picture of the seminatural environment. **B**. The division in zones.

anxiety in standard procedures in rats (Cruz et al., 2015) and mice (Li et al., 2010). The anxiolytic effects seem to be potentiated by estradiol treatment in female rats (Escribano et al., 2014).

3. Thirty-five chocolate pellets (1 g each) (Supreme Mini-Treats; Bio Serv, Frenchtown, NJ) placed on a Petri dish (diameter 100 mm) in the middle of the open area for 30 min. Chocolate is known to be rewarding for rats (see for example Lampert et al., 2013), and its consumption leads to positive affect (Reynaert et al., 2016). Moreover, estrogens enhance the hedonic response to and the consumption of chocolate (Boswell et al., 2006; Lampert et al., 2013; Reynaert et al., 2016).

4. White noise produced by a noise generator (Lafayette instruments, Lafayette, IN) at 90 dB for 15 min. Loud noise produces strong fear responses in rats and is a common stressor used in pharmacological and behavioral studies (see for example Weyers et al., 1994).

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. Predator odor is aversive to rodents (Fendt et al., 2005). Notably, TMT has specific fear-inducing properties in rats (Endres et al., 2005).

These five emotion-inducing events were presented in the above order to all experimental subjects. There was an interval of 50 min between each event, which should have been sufficient for the effects of the previous event to dissipate (see Le Moëne and Ågmo, 2018, for detailed argument for this supposition). The duration of each event has been shown to be long enough for inducing full behavioral effects (lavender, Shaw et al., 2007; music, Escribano et al., 2014; Chikahisa et al., 2007; chocolate, Boswell et al., 2006; white noise, Weyers et al., 1994; fox odor, Endres et al., 2005).

In the wild, rats can be expected to be subjected to a series of disturbances (predators, loud noises, sudden lights, etc.), mixed with positive events (encounter with a mate or with tasty food) in rapid succession during the active period, i.e. the night. Thus, by introducing a series of events rather than an isolated event, we enhanced the external validity of the procedure.

2.5. Procedure

The rats were introduced into the seminatural environment on day 0 at 1 pm. Previously the floor of the entire seminatural environment had been covered with wood chips (Tapvei, Harjumaa, Estonia). Wooden sticks, nest material and small shelters were also provided in the open area. Four 0.5 l bottles of water and about 3 kg of standard food pellets were available in that area. Prior to the introduction, the rats were weighed and marked for identification purposes. After each experimental session, the entire environment was cleaned and disinfected.

The rats were left undisturbed for the first 5 days in the seminatural environment. On day 5, the females were captured and injected with EB. On day 7, all females received P. Three hours and a half later, females were injected with chlordiazepoxide, yohimbine, or saline. Half an hour later, the sequence of emotion-inducing events started. The experiment was terminated after the last emotion-inducing event.

2.6. Design

Each group in the seminatural environment consisted of 4 females and 3 males. One or two females in each group were injected with chlordiazepoxide, yohimbine or saline until a total of 11 females had received each drug, and 10 had received saline. A total of 8 groups were used in this experiment.

2.7. Behavioral observations

Based on our previous studies in the seminatural environment (Chu and Ågmo, 2014, 2015; Snoeren et al., 2015; Le Moëne and Ågmo, 2018), detailed observation for 15 min is sufficient to assess the differences between the different emotion-inducing events and the treatments. We observed the last 15 min of exposure to lavender odor, music and fox odor, since these stimuli require some time to be effective (see Section 2.4 and references therein). The first 15 min of exposure to chocolate, as well as the whole 15 min period of exposure to white noise were observed. These events have immediate effects, and the initial response to these stimuli is of more interest than the sustained response. The ethogram established by Le Moëne and Ågmo (2018) was used in this study (Table 1). The behavioral scoring was realized with the

Table 1
Definition of recorded behaviors. f = frequency; d = duration; l = latency; o = occurrence.

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.
Prosocial behaviors	Resting with other females; f,d	Rests immobilized in relaxed position at a distance shorter than one rat from one or several females.
	Resting with males; f,d	Rests immobilized in relaxed position at a distance shorter than one rat from one or several males.
	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.
	Hiding with another rat[b]; f,d	Immobilized in a corner or in a nest box within one body length of the other rat.
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.
Non-social 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.
	Selfgrooming and scratching; f,d	Self-explanatory.
	Hide alone[b]; f,d	Immobilized in a corner or nest box at a distance longer than one body length to another rat.
	Approach to chocolate[a]; f,l	Coming 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	Grabbing chocolate with paws or mouth.
	Eating[a]; f,d	Chews on chocolate.
	Freezing[b]; f,d	Immobilized in rigid position without any movement including those of vibrissae.
	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.
Exploratory behaviors and ambulatory activity	Sniffing the floor, f,d	Sniffs the floor material with all four paws on the floor.
	Sniffing the nozzles, f,d	Sniffs the air coming out of the nozzles in the wall connected to the odor distribution system.
	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.

[a] Behavior observed only in the presence of chocolate.
[b] Behavior only observed during exposure to white noise.

Observer XT 12.5 program (Noldus, Wageningen, The Netherlands). We recorded the frequency and, whenever possible, the duration of each behavior, its initiator and receiver, as well as the zone of the semi-natural environment in which the behavior was performed.

In addition, we calculated the number of transitions between the zones of the seminatural environment, as well as the time spent in each zone. The lordosis quotient (LQ, number of lordoses displayed/number of mounts received) was also calculated. When a female displayed lordosis in absence of male mounting, the LQ was larger than 1. Finally, latencies to sniff the lavender odor, to approach the plate of chocolate, to flee the noise and to hide from it, and the latency to sniff the fox odor were calculated. Individuals not displaying the behavior were assigned a latency of 900 s, the duration of the observation.

2.8. Co-occurrence analysis

The behavioral observation for each individual produced a list of behaviors in chronological order. This made it possible to determine treatment- or event-induced modifications of the patterning of behavior. We performed co-occurrence analyses using the software Iramuteq (Interface de R pour les Analyses Multidimensionnelles de Textes et de Questionnaires; available at http://www.iramuteq.org/). Raw data for the analysis included several items: treatment of the individual performing the behavior, performed behavior, and emotion-inducing event during which the behavior was displayed. Using a moving window of four items, we determined how often one behavior pattern occurred together with another in the same window. This is defined as a co-occurrence. The procedure to analyze the data is detailed in Le Moëne and Ågmo (2018). Briefly, descending hierarchical classification was used in order to find clusters of related behaviors (Reinert, 1983; Reinert, 1990; Valax et al., 1990, see also LePape et al., 1997). Clusters illustrate the probability for items to co-occur significantly more often together than with items of another cluster, as evaluated by χ^2 analyses

(Marchand and Ratinaud, 2012). Clusters can be interpreted as groups of behaviors occurring significantly closer in time than other behaviors in the repertoire, therefore defining coherent behavioral sequences.

2.9. Statistical analysis

2.9.1. Analysis of the effect of emotion-inducing events

The behaviors identified as characteristic for a treatment or an emotion-inducing event in the co-occurrence analysis were evaluated. To determine the effect of the emotion-inducing events, we compared the target event to the mean of the four others. The aim of the comparison was to determine whether one emotion-inducing event indeed differed from the others. Therefore, depending on the event regarded, the mean is not calculated from the same four other events. Because of this, the data are represented as the mean percentage of difference between the evaluated event and the mean of the other events [(value of the event E) − (mean of the 4 other events) / mean of the 4 other events].

To assess the effect of each event compared to the mean we used a one sample *t*-test, of which the P-value, when significant, was adjusted with the Bonferroni correction. When the use of the one sample *t*-test was not possible, we used the Wilcoxon one-sample test, followed by the Bonferroni correction. All P-values reported in the results have been modified with the Bonferroni correction corresponding to the number of comparisons made. The nominal significance threshold was set to P < 0.05.

2.9.2. Analysis of the effect of the treatment

The effect of the treatment was first analyzed over the course of the whole experiment (all events collapsed). If a significant effect was found, we then proceeded to analyze the effect of the treatments within each event separately. Only differences from the control group treated with saline are reported. When the data permitted it, we used a two-

way ANOVA with event as within groups factor and treatment as between groups factor, followed by a post hoc Tukey HSD test. When the data deviated from the normal distribution according to Shapiro-Wilk's test, or the error variances were non-homogenous according to Hartley's Fmax test, we used a Kruskal-Wallis test followed by the post hoc Conover test. The number of individuals fleeing the noise at its onset was analyzed through a Chi-squared test. The significance threshold was P < 0.05. All tests were performed using the IBM SPSS Statistics, version 24 and R, version 3.4.3 (core, lsr, PMCMRplus and effsize packages).

3. Results

3.1. Effects of emotion-inducing events

Since the analysis of the effect of the emotion-inducing events could have been confounded by the effect of the treatments, we first analyzed the effect of the events exclusively on our control group. However, since the differences between the analyses of saline-treated animals and those including all subjects regardless of treatment were marginal, we only present the results obtained from the latter analyses.

3.1.1. Co-occurrence analysis (Fig. 2)

An analysis of co-occurrence allowed to distinguish 3 clusters of behaviors and emotion-inducing events (Fig. 2). The lavender event was distinguishable by its association with the behavior "drinking" and

the sexual behaviors. The chocolate event was associated with all social behaviors, both prosocial and antisocial ones, as well as with the sexual behavior "rejection". The white noise event was associated with the exploratory behaviors "sniffing the floor" and "rearing", as well as with the non-social behaviors "self-grooming" and "resting alone". The events music and fox odor were merged with the white noise and did not present a salient profile. Therefore, we will focus our analysis on the behaviors during each of the 3 other emotion-inducing events defined by the co-occurrence analysis, being lavender odor, chocolate and white noise. Only the behaviors observable during all emotion-inducing events were included in the analysis. Behaviors that were observable for only one event (e.g. flee the noise) were not included here.

3.1.2. Specific behaviors associated with of the exposure to lavender odor (Fig. 3)

The exposure to lavender modified sexual behaviors. The lordosis frequency was higher than the mean of the four other conditions [$t_{(31)} = 3.213$, P = 0.028], so was the LQ [$t_{(31)} = 3.254$, P = 0.025] and the frequency of paracopulatory behaviors [$t_{(31)} = 4.008$, P = 0.003]. In parallel to female sexual behavior, male mount frequency significantly increased during exposure to lavender compared to the mean of the other conditions [$t_{(31)} = 2.401$, P = 0.046], as did the frequency of pursuing females [$t_{(31)} = 3.466$, P = 0.004] (data not shown).

Exposure to lavender increased the frequency of antisocial behaviors directed to other females. The nose-off frequency against other
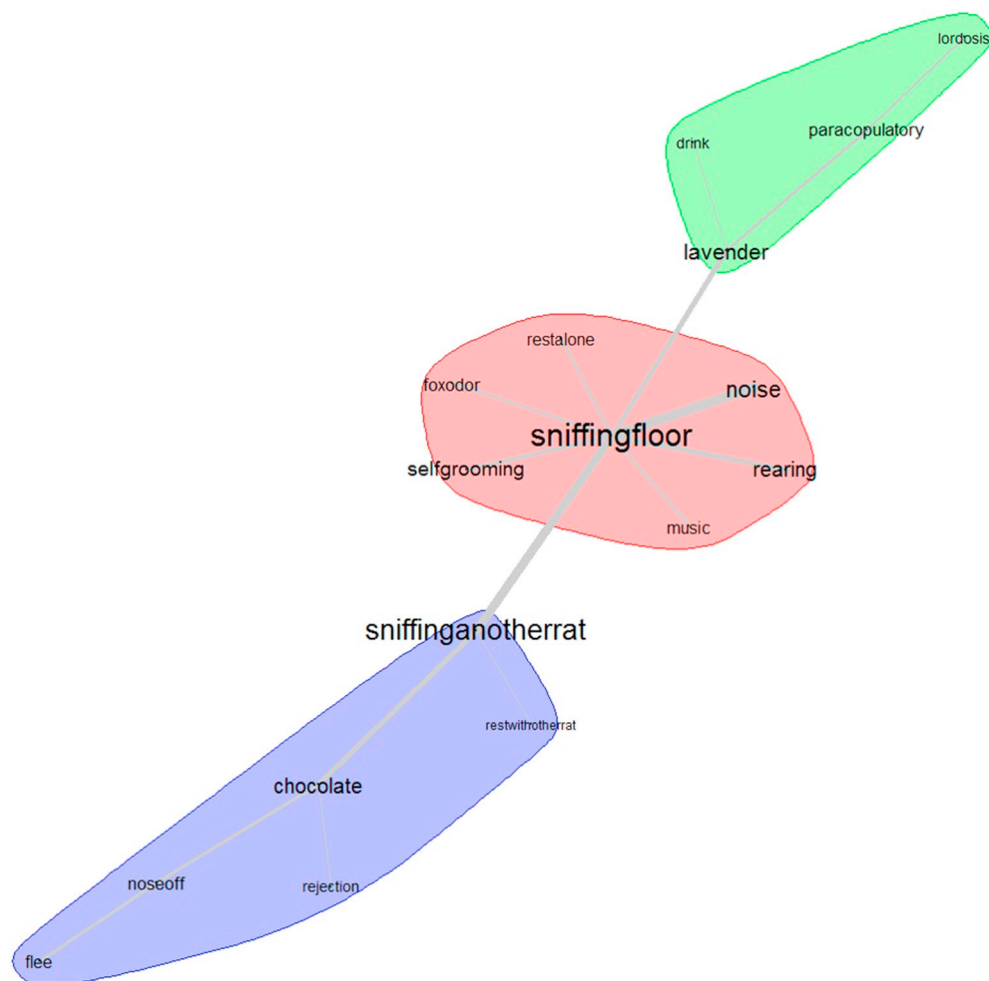

Fig. 2. Co-occurrence analysis showing main behavioral associations typical of each of the experimental conditions over the course of the entire sequence of emotion-inducing events (excluding event-specific behaviors). Clusters of behavioral association are represented in halos of different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
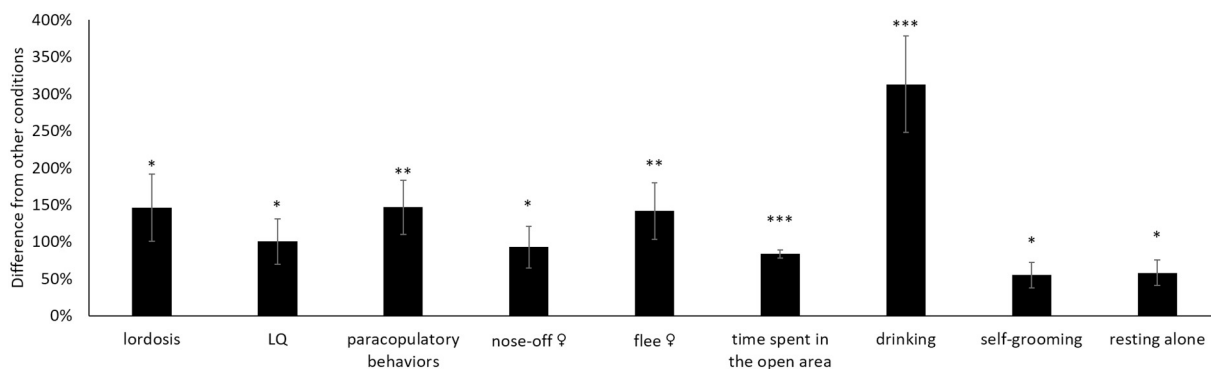
Fig. 3. Effect of exposure to lavender odor on female behavior. Only behaviors significantly altered by the exposure to lavender compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.

females was higher than the mean of the other conditions [$t_{(31)} = 3.334$, P = 0.020] but not against males [$t_{(31)} = 0.875$, P = 0.388]. This was also the case for the frequency of fleeing from another female [$t_{(31)} = 3.694$, P = 0.008] but not for the frequency of fleeing from the males [$t_{(31)} = 2.039$, P = 0.451].

Finally, The time spent in the open area was increased [$t_{(31)} = 14.505$, P < 0.001], and non-social behaviors were also more frequent during exposure to lavender. The females drank more often [$t_{(31)} = 4.791$, P < 0.001], displayed a higher frequency of self-grooming episodes [$t_{(31)} = 3.180$, P = 0.030], and rested alone more frequently [$t_{(31)} = 3.398$, P = 0.020].

3.1.3. Specific behaviors associated with of the exposure to chocolate (Fig. 4)

Exposure to chocolate decreased the social behavior "resting with another rat" compared to the mean of the other conditions [$t_{(31)} = 8.000$, P < 0.001], but increased the frequency of sniffing the males [$t_{(31)} = 3.092$, P = 0.046]. The frequency of sniffing the other females was unchanged [$t_{(31)} = 2.273$, P = 0.332].

Higher antisocial behaviors were observed during exposure to chocolate. The females displayed a higher number of nose-off episodes to both sexes [females: $t_{(31)} = 3.069$, P = 0.049; males: $t_{(31)} = 4.002$, P = 0.004]. The same was observed with the frequency of fleeing from other rats [females: $t_{(31)} = 3.977$, P = 0.008; males: $t_{(31)} = 4.412$, P = 0.001].

The olfactory investigation of the seminatural environment was decreased during exposure to chocolate. The females sniffed the floor less compared to the mean of the other conditions [$t_{(31)} = 4.223$, P = 0.002] and displayed a lower rearing frequency [$t_{(31)} = 3.555$,

P = 0.014]. The presence of chocolate increased the number of transitions between the burrow and the open areas [$t_{(31)} = 7.313$, P < 0.001] and the time spent in the open area [$t_{(31)} = 6.052$, P < 0.001]. Consequently, the time spent in the burrow was decreased [$t_{(31)} = 8.776$, P < 0.001].

3.1.4. Specific behaviors associated with exposure to white noise (Fig. 5)

Exposure to white noise decreased sexual behaviors compared to the mean of the other conditions. This was the case for the lordosis frequency [$t_{(31)} = 21.707$, P < 0.001], the LQ [$t_{(31)} = 11.178$, P < 0.001] and the frequency of paracopulatory behaviors [$t_{(31)} = 30.589$, P < 0.001]. Also, male sexual behavior was inhibited during exposure to white noise. The mount frequency decreased compared to the mean of the other conditions [$t_{(31)} = 29.451$, P < 0.001], and so did the frequency of pursuing females [*Wilcoxon one-sample* $_{(31)} = 5.657$, P < 0.001] (data not shown).

The prosocial behavior "sniffing another rat" was increased both when directed to other females [$t_{(31)} = 5.143$, P < 0.001], and to males [$t_{(31)} = 5.398$, P < 0.001]. To the contrary, the frequencies of fleeing from other females and from males were both decreased [females: *Wilcoxon one-sample* $_{(31)} = 5.657$, P < 0.001; males: $t_{(31)} = 21.971$, P < 0.001].

White noise increased the olfactory investigation of the seminatural environment. The frequency of sniffing the floor increased [$t_{(31)} = 7.892$, P < 0.001], and so did the rearing frequency [$t_{(31)} = 4.000$, P = 0.005]. The number of transitions between the open area and the burrow decreased [$t_{(31)} = 4.852$, P < 0.001], and the time spent in the open area was shorter [$t_{(31)} = 125.988$, P < 0.001]. To the contrary, the time spent in the burrow was longer than the mean
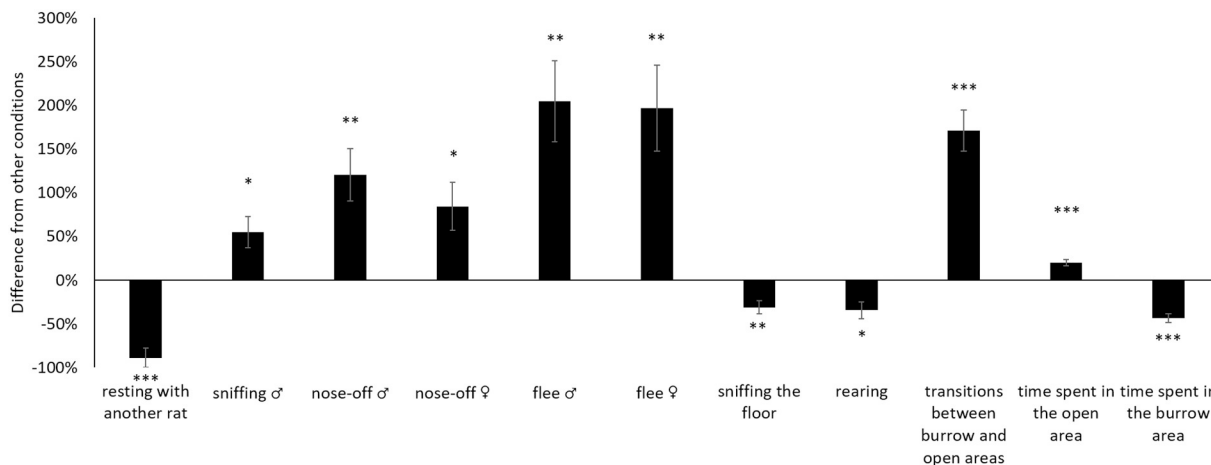

Fig. 4. Effect of exposure to chocolate pellets on female behavior. Only behaviors significantly altered by the exposure to chocolate compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.
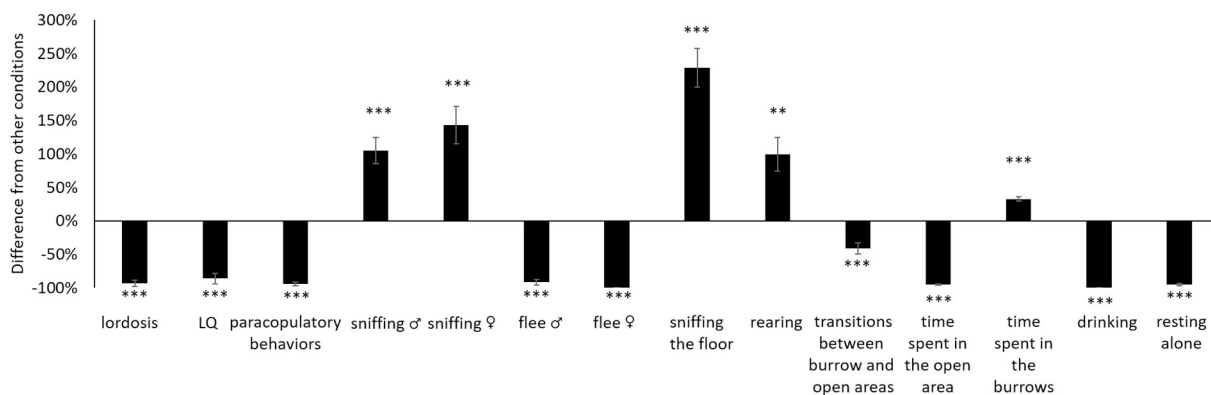
Fig. 5. Effect of exposure to white noise on female behavior. Only behaviors significantly altered by the exposure to white noise compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.

of the other conditions [$t_{(31)}$ = 9.678, P < 0.001]. Finally, exposure to white noise reduced the drinking frequency [*Wilcoxon one-sample* $_{(31)}$ = 5.657, P < 0.001] and the frequency of resting alone [$t_{(31)}$ = 58.713, P < 0.001].

3.2. Effects of treatments

3.2.1. Co-occurrence analysis

We analyzed the co-occurrence of females' behaviors according to the treatment of the individual initiating the behavior, all emotion-inducing events collapsed, and including behaviors that were specific of each event (Fig. 6). The yohimbine-treated group formed an independent cluster with the sexual behaviors and behaviors associated with chocolate exposure. The antisocial behaviors fleeing and nose-off

were also characteristic of this group. The treatment groups saline and chlordiazepoxide belonged to the same cluster, associated with prosocial behaviors, behaviors specific to white noise avoidance and the olfactory exploration of lavender and fox odor.

We then proceed to look at the co-occurrence of females' behaviors according to the treatment of the individual initiating the behavior, under each emotion-inducing event. Since music, white noise and fox odor all belonged to the same cluster in the previous analysis, these events were collapsed into "aversive conditions" for the analysis of the treatment (Fig. 7).

During the exposure to lavender, each treatment group could be isolated in an independent cluster. The saline group was associated with resting alone and the most prominent exploratory behavior "sniffing the floor". The chlordiazepoxide group was mainly linked to the prosocial
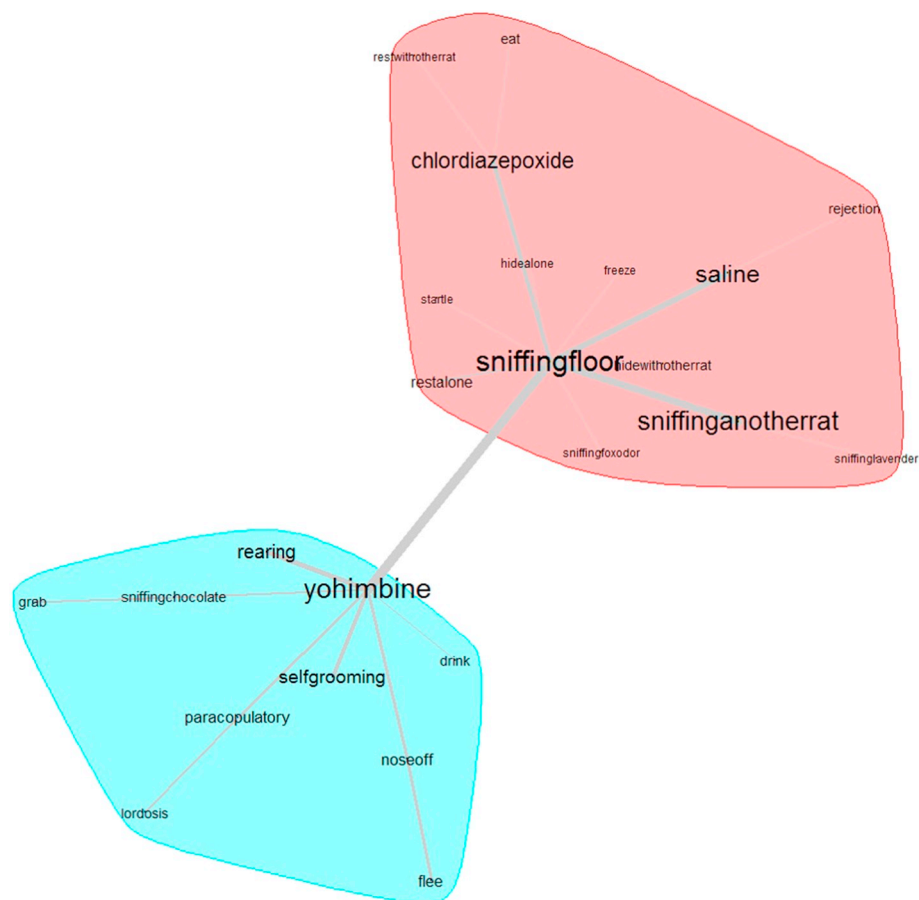
Fig. 6. Co-occurrence analysis showing main behavioral associations typical of each of the treatments, over the course of the entire sequence of emotional challenges (including event-specific behaviors). Clusters of behavioral association are represented in halos of different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. Co-occurrence analysis showing main behavioral associations typical of each of the emotion-inducing event. **A.** Lavender odor, **B.** Chocolate, **C.** Aversive conditions (music, white noise and fox odor). Clusters of behavioral association are represented in halos of different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
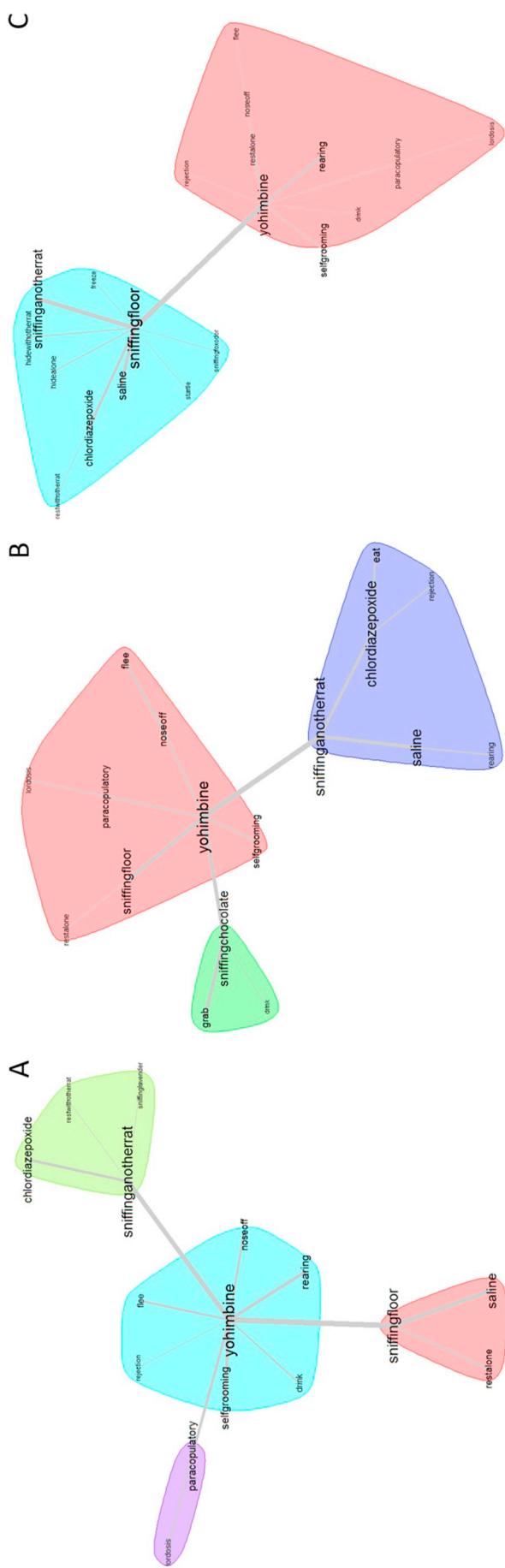
behavior "sniffing another rat" and to the exploratory investigation of the lavender odor. The group treated with yohimbine was associated with anti-social behaviors, the exploratory behavior "rearing" and the non-social behavior "self-grooming". In addition, the cluster characteristic of yohimbine treatment was associated with a separated cluster of sexual behaviors (Fig. 7A).

During exposure to chocolate, the saline and the chlordiazepoxide groups belonged to the same cluster. They were mainly associated with the prosocial behavior "sniffing another rat", and with some exploratory and chocolate-specific behaviors. The yohimbine-treated group was isolated in a separate cluster, associated with sexual, anti-social and exploratory behaviors. Additionally, it was linked to a distinctive cluster containing most chocolate-specific behaviors (Fig. 7B).

During exposure to aversive events, the chlordiazepoxide- and saline-treated groups belonged to the same cluster. Most fear-related behaviors were found in their cluster, as well as the exploratory behavior "sniffing the floor" and the prosocial behavior "sniffing another rat". Yohimbine belonged to another cluster with sexual, antisocial and non-social behaviors, as well as the exploratory behavior "rearing" (Fig. 7C).

3.2.2. Treatment effects on specific behaviors
3.2.2.1. Treatment effect, all emotion-inducing events collapsed. We had predicted that the anxiogenic properties of yohimbine would inhibit sexual behaviors, and enhance fear-related behaviors in all emotion-inducing events. In addition, we hypothesized that chlordiazepoxide would enhance sexual behaviors in fear-inducing events. Chlordiazepoxide should be effective only in fearful events, and have no effect in pleasant situations. When all emotion-inducing events were collapsed, the LQ was altered by the drug treatment [$H_{2, N=32}$ = 7.157, P = 0.028]. Contrary to what we expected, the LQ was higher for females treated with yohimbine than females treated with saline [P = 0.025]. Chlordiazepoxide was ineffective [P = 0.783] (Fig. 8A). Treatment also affected to frequency of self-grooming [F(2, 31) = 7.321, P = 0.003]. The yohimbine-treated group displayed self-grooming episodes more often than the saline group [P = 0.046]. We found no effect of chlordiazepoxide [P = 0.490] (Fig. 8B).

3.2.2.2. Treatment effects under the different emotion-inducing events. When evaluating treatment effects within each of the emotion-inducing event, it was found that the LQ differed between treatments only during exposure to lavender odor [$H_{2, N=32}$ = 7.776, P = 0.020]. The treatment did not modify the LQ during chocolate exposure nor during aversive events [P > 0.447]. During exposure to lavender, yohimbine treatment increased LQ compared to saline [P = 0.008], while we found no effect of chlordiazepoxide [P = 0.720] (Fig. 9). None of the other behaviors observed showed an effect of the interaction treatment*event (data not shown).

The behavior "hiding" was specific to the white noise event. It was not observed under any other event. The frequency of hiding alone was affected by the treatment [F(2, 31) = 5.103, P = 0.013]. The females treated with chlordiazepoxide hid alone more frequently than those treated with saline [P = 0.021]. Yohimbine did not differ from saline [P = 0.956] (Fig. 10A). The latency to hide alone was also affected by the treatment received [F(2, 31) = 3.973, P = 0.030] and the latency was shorter for females treated with chlordiazepoxide than for females treated with saline [P = 0.027], while the females treated with yohimbine were not different [P = 0.668] (Fig. 10B). All females fled the noise with equal probability [χ_2^2 = 1.130, P = 0.568] (Fig. 10C), but with a different latency [$H_{2, N=32}$ = 6.530, P = 0.038]. The females treated with yohimbine had a shorter latency to flee the noise than the females treated with saline [P = 0.032]. The females treated with chlordiazepoxide did not differ from the control group [P = 0.829] (Fig. 10D). The treatment had no effect on the other observed behaviors. Behaviors specific of the other events were not altered by the drug treatments either.
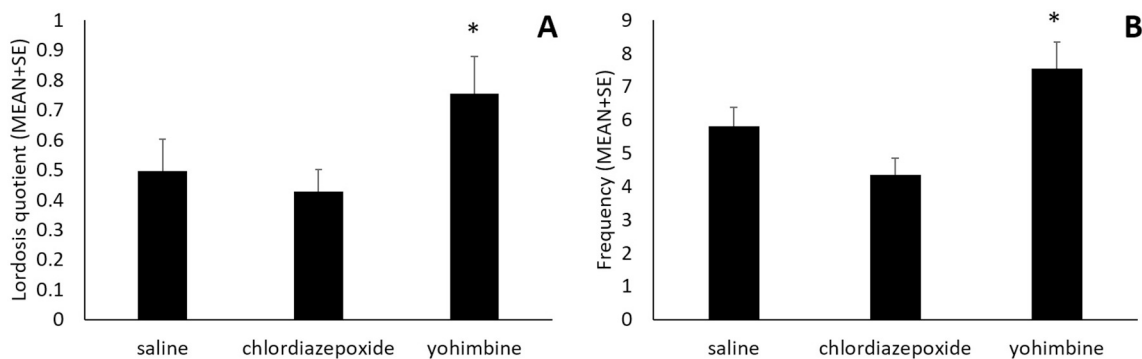
Fig. 8. A. Effect of the treatment on lordosis quotient (all experimental condition collapsed). **B**. Effect of the treatment on self-grooming (all experimental condition collapsed). Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: n = 10, chlordiazepoxide: n = 11, yohimbine: n = 11.
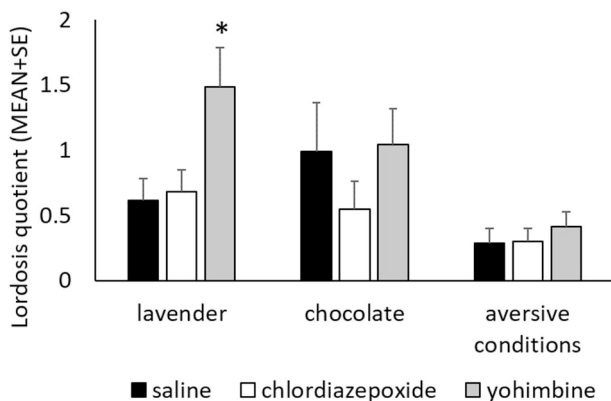

Fig. 9. Effect of the interaction between the treatment and the experimental condition on the lordosis quotient. Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: n = 10, chlordiazepoxide: n = 11, yohimbine: n = 11.

4. Discussion

4.1. Effect of the emotion-inducing events

In the present study, we found that the different emotion-inducing events were able to elicit different behavioral patterns, probably caused by different emotions. The analyses of co-occurrences highlighted that 3 clusters of behavioral patterns could be distinguished: one for lavender odor, one for chocolate exposure, and one for white noise, fox odor and the Mozart sonata. Based on data from the literature, we had predicted that this particular piece of music played here would induce positive affect. However, during the course of the present study, we obtained data from another experiment showing that the music rather had aversive properties in the seminatural environment (Le Moëne and Ågmo, 2018). The sound level (55–60 dB) is similar to that used in other studies reporting anxiolytic effects of the same Mozart sonata (ranging from 50 to 75 dB, see for example Chikahisa et al., 2007; Lu et al., 2010 or Escribano et al., 2014). One possible explanation for the unexpected effect of music is that in the seminatural environment, the music was suddenly introduced into a well-known, quiet environment. This is very different from the earlier studies, in which the subjects were transferred to a new situation and tested in an unknown procedure, of which music was a part. Perhaps music can reduce reactions to novelty, whereas it is disruptive in familiar environments. This hypothesis remains to be confirmed, but it can at least be tested experimentally.

The analysis of separate behavioral items revealed that lavender odor increased sexual behaviors, and chocolate exposure increased social behaviors. Both emotion-inducing events increased the time spent in the open area. To the contrary, exposure to white noise decreased

sexual behaviors and altered classical indices of fear, for example avoidance of the open area.

It cannot be excluded that the order in which the emotion-inducing events were presented could have impacted the results obtained. Even though a 50-min interval was imposed between events, some effect might have been carried over to the next event. Only further studies could determine if this indeed was the case. It must be observed that male sexual behaviors were modified in the same way as female behaviors. However, the changes in male behavior cannot explain the increase in LQ observed during lavender exposure or the reduced LQ observed during white noise. The LQ is an indicator of female receptivity independent of male behavior. With regard to the effects of the emotion-inducing stimuli on sexual behavior, it might be maintained this behavior had changed during the long observation period, and that this change could confound the results. However, we have previously shown that the hormone treatment used here maintain female sexual behavior at a stable level for 6.35 ± 0.42 h (mean ± SEM, Le Moëne et al., 2015).

4.2. Effects of treatments on fear-related behaviors

According to our predictions, chlordiazepoxide would be effective only in the emotion-inducing event provoking fear. It is not surprising, then, that this drug was not different from saline when all events were collapsed in the co-occurrence analysis. However, in the events causing fear or an aversive reaction, that is music, white noise and fox odor, chlordiazepoxide and saline appeared in the same cluster. This was contrary to our prediction. When particular behavioral items were analyzed, a slightly different image emerged. Chlordiazepoxide did not affect any behavioral item in any event except during white noise, when this drug reduced the latency to hide alone and increased the frequency of that behavior. It is not evident that these effects represent an anxiolytic action. However, it was previously shown that stressed rats actively engage in social behavior more often than non-stressed rats (Taylor, 1981). By hiding alone during white noise, chlordiazepoxide-treated females engaged in less social contact than saline-treated females. This could be interpreted as an anxiolytic effect of chlordiazepoxide. Interestingly, only during exposure to lavender, which elicited a positive affect, chlordiazepoxide and saline belonged to separate clusters. Contrarily to what we observed during white noise, chlordiazepoxide was then strongly associated with prosocial behaviors. This is consistent with previous findings showing that in non-aversive events, chlordiazepoxide increases the motivation to interact with a conspecific (File and Hyde, 1978; Bonuti and Morato, 2018).

We had also predicted that yohimbine would enhance fear-related behaviors in all emotion-inducing events. Yohimbine indeed emerged with a salient profile during both positive events, lavender and chocolate, and during the aversive events.
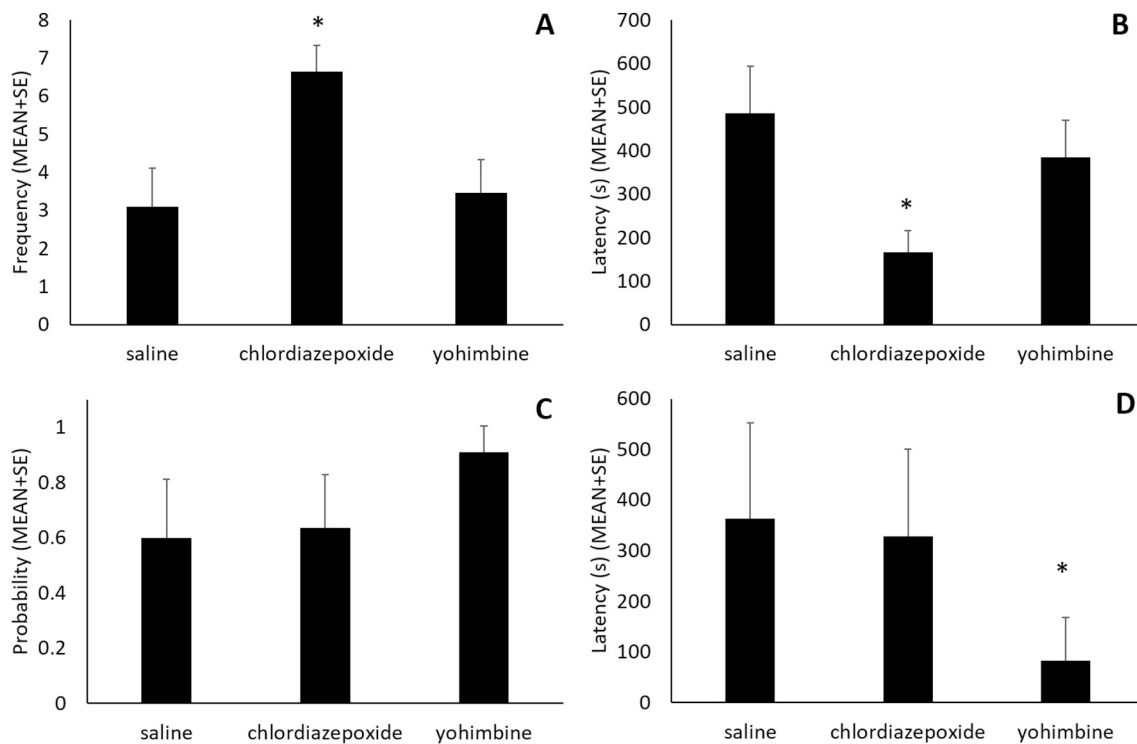
Fig. 10. Effect of the treatment on white noise-specific behaviors. **A**. Frequency to hide alone. **B**. Latency to hide alone. **C**. Probability to flee the noise at its onset. **D**. Latency to flee the noise at its onset. Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: n = 10, chlordiazepoxide: n = 11, yohimbine: n = 11.

4.3. Effects of treatments on sexual behavior

An unexpected effect of yohimbine was the enhanced sexual receptivity displayed by the females. This effect became evident during exposure to lavender odor, even though the LQ was significantly increased also when all emotion-inducing events were collapsed. Earlier studies had shown that yohimbine failed to stimulate sexual behavior in intact, estrous females (Ventura-Aquino and Fernández-Guasti, 2013) and in ovariectomized females treated with estradiol + progesterone (Clark et al., 1985). A contradictory observation was made in rats primed with a low dose of estradiol without subsequent progesterone treatment (Everitt et al., 1975). Whether yohimbine might be of any therapeutic use in the human clinic is uncertain, however. Human studies have given largely negative results (Meston and Worcel, 2002; Piletz et al., 1998), and there is currently little interest in further trials of yohimbine as a clinically useful, prosexual drug.

White noise almost eliminated sexual behavior. This effect was not reduced by chlordiazepoxide, something contrary to our predictions. The modest anxiolytic effect observed may not have been large enough to counteract the consequences of the noise, or chlordiazepoxide may have inhibiting actions on sexual behavior by itself, independent of anxiolytic actions. To our knowledge, the effects of benzodiazepines on female sexual behavior have not been studied. In the human, data are inconclusive (La Torre et al., 2014; Clayton et al., 2016) The most likely explanation for the incapacity of chlordiazepoxide for reducing the deleterious effects of white noise on sexual behavior is, in fact, a combination of insufficient anxiolytic action and the noise-induced suppression of sexual activity in the males.

4.4. Potential factors limiting the effect of the drugs

In general, we found few effects of treatment in the present study. One reason for this may be that the doses used here were sub-effective. The establishment of a dose-effect relationship in the seminatural environment would have confirmed or rejected this hypothesis. However,

the inevitable sedative effects of larger doses would have made the interpretation of any behavioral changes difficult. Both chlordiazepoxide and yohimbine have considerable sedative effects in doses slightly above those used here (Ågmo and Fernández, 1991; Viitamaa et al., 2006). It could also be argued that the drugs used were effective only during a part of the long observation period (5.5 h). However, chlordiazepoxide has a half-life in rats of about 4–6 h (Koechlin and d'Arconte, 1963) whereas the corresponding value is about 7–8 h for yohimbine (Hubbard et al., 1988). There are also studies reporting long-lasting behavioral and physiological effects of these compounds. Chlordiazepoxide modified rats physiological indices up to 7.5 h post-injection (e.g. arterial blood pressure and body temperature; Froger-Colléaux et al., 2011), and reduced motor activity for many hours (Randall et al., 1960; Froger-Colléaux et al., 2011). Yohimbine showed several effects on stress-related neuro-hormonal and metabolic response and modified rats' behavioral responses for at least 5 h post-injection (Ambrisko and Hikasa, 2003; Figlewicz et al., 2014). Moreover, in the present study, some effects of the treatments were observed during the first as well as during the last emotion-inducing events, confirming the long lasting effects of these compounds. This strongly suggests that neither dose nor duration of observation was the cause of the small effects observed, at least not the main cause.

A more likely explanation for the modest effects of chlordiazepoxide and yohimbine on fear related behaviors is the social context. In the seminatural environment, the phenomenon called social buffering might be of importance. This concept refers to the reduced manifestations of fear when the experimental subject is exposed to distressing stimuli while in the company of a conspecific (reviewed in Kiyokawa and Hennessy, 2017). Social buffering has been well documented in studies of conditioned fear responses, even in female rats. It is independent of the phase of the estrus cycle, consequently also of the ovarian hormones (Ishii et al., 2016). Moreover, the greater the number of conspecifics present, the greater the social buffering effect (Kiyokawa et al., 2018). Thus, this effect could be considerable in the seminatural environment, in which 6 conspecifics are present. There are reports

showing that olfactory stimulation from conspecifics is necessary and sufficient for social buffering to occur (reviewed in Kiyokawa, 2017). Since conspecific odor should be present in the entire seminatural environment, it is possible that potential anxiety responses to yohimbine treatment were attenuated to the degree of becoming undetectable. Likewise, potential effects of chlordiazepoxide could have been masked by the anxiolytic effects of the social context. It can be observed that a typical response to white noise, freezing (e.g. Koba et al., 2016; Yoshimoto et al., 2010), was almost absent in the seminatural environment. This could be an example of the consequences of the social buffering. Moreover, freezing behavior was absent in mice submitted to a fearful stimulus, when they had the possibility to take shelter (Vale et al., 2017). The presence of conspecifics, as well as the possibility to reach for safety, both features available in the seminatural environment, could modulate classical indices of fear.

Another factor contributing to the modest anxiogenic effect of yohimbine could be the females' possibility to express a large variety of responses to the emotion-inducing events. This should enhance the perceived controllability, which in turn is known to reduce the intensity of stress and fear responses in a variety of conditions, at least in male rats (Anisman and Zacharko, 1982; Kant et al., 1991). Unfortunately, controllability has recently been reported to fail to modify stress responses to conditioned fear in female rats (Baratta et al., 2018). Whether this is the case also for unconditioned fear reactions remains unknown. Furthermore, the importance of controllability has been firmly established for conditioned stress and fear responses, but evidence is less clear with regard to the immediate reaction to a fearful stimulus (Baratta et al., 2007; Kant et al., 1991). It is not impossible, though, that controllability may contribute to the almost absent effects of yohimbine on fear-related behaviors in all emotion-inducing events.

4.5. On the utility of the seminatural environment for evaluating responses to emotional challenges and the actions of anxiolytic or anxiogenic drugs

The modest effects of the drugs, in doses known to be effective in other procedures (see Material and methods), can perhaps be consequences of social buffering and controllability, as mentioned. However, the power of these phenomena must be limited, since they did not impede behavioral manifestations of fear in the aversive contexts (present data, Blanchard and Blanchard, 1989). Attention should be drawn to the fact that some of the drug effects were visible despite the compensatory mechanisms implemented by the rats. It should also be observed that when the effects of anxiolytic compounds are studied in simpler procedures, for example an open field with a solitary experimental subject, chlordiazepoxide affects only a small fraction of the subject's behavioral repertoire (Choleris et al., 2001). It should also be noted that several benzodiazepines, including chlordiazepoxide, were found to be almost inactive in a fear-and-defense-reaction test battery incorporating ethologically relevant stimuli (Blanchard et al., 1989). Finally, it is possible that the actions of anxiolytic or anxiogenic compounds become evident only in specific test procedures exclusively designed for showing effects of such drugs. The limited success of some animal models in neuropsychiatric research (e.g. Belzung, 2014) has prompted a search for ethological approaches in preclinical drug studies (Peters et al., 2015) and the use of seminatural conditions has some enthusiastic advocates (e.g. Zilkha et al., 2016; Weissbrod et al., 2013). The present study should inspire caution with such proposals.

In conclusion, present data confirm that emotion-inducing events introduced into a seminatural environment do modify the subjects' behavior in predictable ways. They also show that the effects of anxiolytic and anxiogenic drugs are attenuated in social contexts and in an environment in which the experimental subjects can express a considerable proportion of their natural behavioral repertoire. Nevertheless, the drugs retain most of their basic behavioral actions.

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