Department of Psychology

Estrogenic modulation of socio-sexual and fear-related behaviors in female rats:

Properties of the estrogen receptors α and β in a procedure with external validity

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Contents

Abstract

A number of psychiatric troubles are distributed along a biased sex ratio. Differences in sex steroids levels, notably estradiol, could account for this bias. Differential expression and activation of the two known estrogen receptors (ER), α and β could result in different behavioral patterns. Indeed, these two receptors play an important, but unequal, role in the regulation of socio-sexual and fear-related behaviors. First, I ethologically characterized anxiety-related behaviors in adult female rats. Then, I systematically administered ER agonists to observe the role of ERs on behavioral responses and structure. Finally, I evaluated 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 encoded with an adeno-associated virus directed against each of these receptors. All studies were conducted in a seminatural environment in order to obtain externally valid, transferable results. In this environment, several emotion-inducing stimuli were introduced to determinate ERs' involvement on situation-dependent behavioral responses. ERα activation was necessary for the display of lordosis and paracopulatory behaviors in female rats, as well as for their sexual attractivity to males. Expression of ERα in the ventral nucleus of the hypothalamus (VMN) was necessary for lordosis. The receptor in the VMN also showed anxiogenic properties during exposure to white noise. My findings suggest that ERα in the VMN had anxiogenic properties in threatening situations, and facilitated copulation in safe environments. Treatment with ERβ agonist modified behavioral structure during exposure to aversive stimuli, and silencing this receptor in the CeA increased rat anxiety. Therefore, I conclude that ERβ has anxiolytic properties, partly acting through the CeA. Better understanding of the implications of each ER within different brain structures will help unveiling their seemingly opposite roles.

List of papers:

- Le Moëne, O. and Ågmo, A. (2019). Responses to positive and aversive stimuli in estrous female rats housed in a seminatural environment: Effects of yohimbine and chlordiazepoxide. *Pharmacology, Biochemistry and Behavior*, **179**, 43-54.
- Le Moëne, O. and Ågmo, A. (2018). Behavioral responses to emotional challenges in female rats living in a seminatural environment: The role of estrogen receptors. *Hormones and Behavior*, **106**, 162-177.
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1. General introduction

1.1 Psychiatric troubles and sex bias

1.1.1 The global issue of mental illness

The issue of mental health is slowly emerging from the dark closet of shame it has been kept into in the last centuries. Treatment and structures of support are improving fast, as psychiatric troubles are globally becoming more socially acceptable. In this frame, neuroscience shows a great potential for better understanding and caring for people affected by these troubles (Saxena, 2016).

Sex is a significant risk factor for neurodevelopmental and neurodegenerative disorders (Pinares-Garcia et al., 2018). A number of psychiatric disorder are distributed along a biased sex ratio, with two notable examples being autism (3 males for 1 female; Loomes et al., 2017) and depression (1.7 females to 1 male, Whiteford et al., 2013) (Fig. 1). The biological mechanisms at work in sex-specific characteristics of typical or atypical brain have been the subject of intense research. One major difference between sexes is the levels of circulating sex steroids. Their levels and their neurobiological role could account for sex differences in psychiatric disorders. Notably, unbalanced estrogen metabolism can lead to increases in cardiovascular risk factors (Dai et al., 2012), or facilitate the initiation of some types of cancer (Zahid et al., 2013). Restoring estrogen levels can ameliorate severe conditions such as postpartum depression (Ahokas et al., 2001).

Nowadays, the role of sex steroid in brain organization and development, as well as in brain sexual differentiation is well known (e.g. Gillies and McArthur, 2010; McEwen et al., 2017). It is very likely that sex differences in the event of mental illness result from complex interactions between sex hormones and genetic and epigenetic factors, and later refined by cultural and social ones.

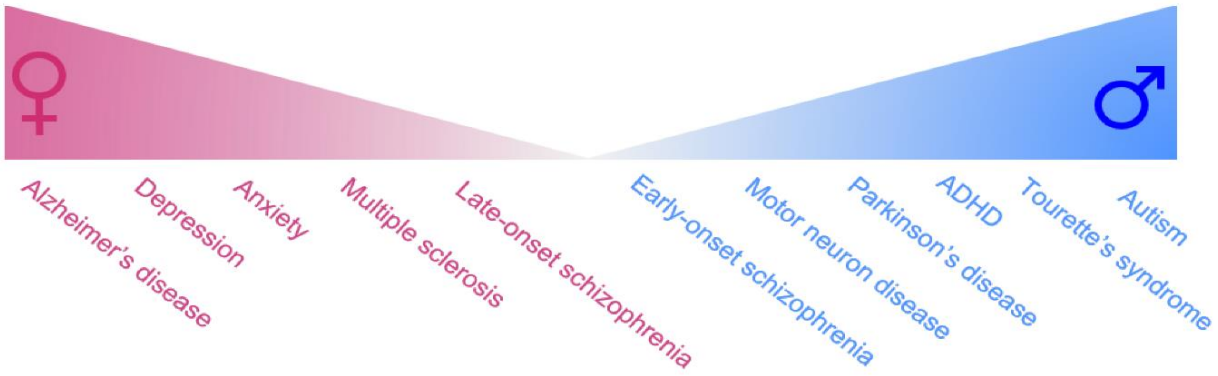

Fig. 1. Gradient in sex differences in the prevalence of neurodegenerative and neuropsychiatric disorders. Abbreviations: ADHD, Attention-deficit hyperactivity disorder. Reproduced with permission from Pinares-Garcia et al., 2018.

1.1.2 Estrogens production and functions

Estrogens circulate in higher levels in females than in males, with estradiol (E2) being the main female sex hormone. Estradiol is secreted in pulses varying across the time of the day and the menstrual cycle (Fig. 2). The levels of estradiol are the lowest in gonadectomized females and peak during proestrus in gonadally intact females (Butcher et al., 1974; Walmer et al., 1992). In addition to the incidence of puberty and the development of sexual characteristics, estradiol fulfills several physiological and behavioral functions. Notably, female socio-sexual behavior depends on hormonal levels. Sexual behaviors are elevated during behavioral estrus, and remain remarkably stable during its entire duration (Chu and Ågmo, 2014). Therefore, the estrus period is a privileged period to study the implications of estrogens in behavioral responses. Besides the regulation of sexual behavior, estrogens modulate aggression levels (Albert et al., 1992; Trainor et al., 2006) and other social behaviors (Hliňáck, 1993; Walf and Frye, 2008). In addition, estrogens play a role in the modulation of stress and anxiety levels (Frye and Walf, 2004; Morgan and Pfaff, 2002, 2001).

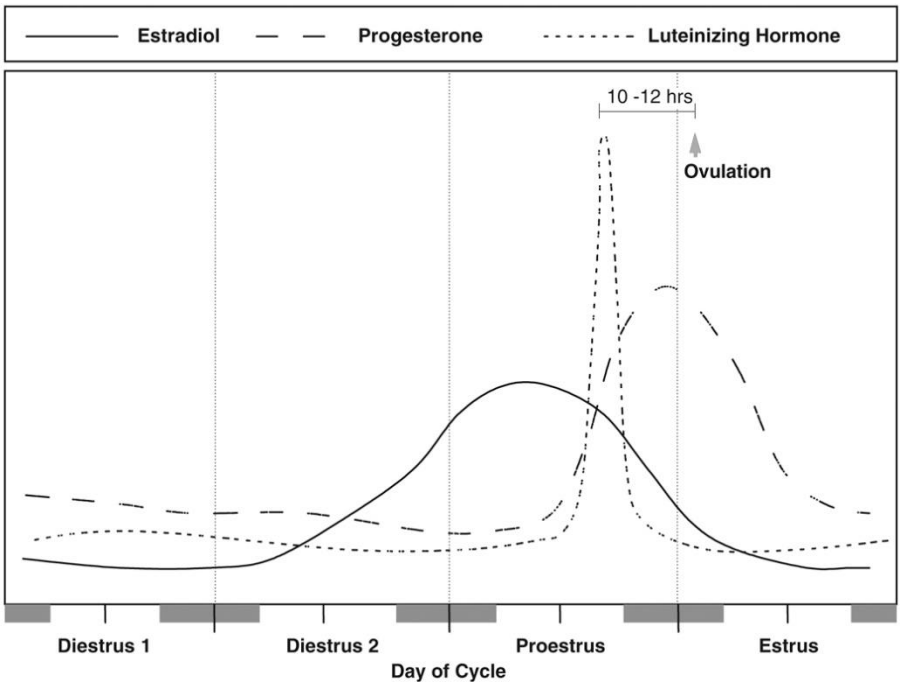

Fig. 2. Four-day estrous cycle in an intact female rat. Reproduced with permission from Goldman et al., 2007.

Following estradiol administration, several studies report increased activity (locomotion, wheel-running) in safe environments such as the home cage, and inhibited locomotion and exploration in novel or unsafe environments such as in the open field or the dark-light transition tests (Morgan et al., 2004; Morgan and Pfaff, 2002; Morgan et al., 2004). Thus, estrogenic actions seem to manifest differently, depending on details of the environmental context. Taken together, the actions of estrogens seem to enhance arousal (Pfaff et al., 2002). Arousal is "a non-specific tonic state of neural activity which modulates not only the sleep/waking cycle, but also the efficiency of performance in the waking state" (Robbins, 1984, pp. 14), and individuals with higher generalized arousal were identified as being "more alert to sensory stimuli of all sorts, more motorically active, and more reactive emotionally" (Pfaff, 2006, pp. 5).

In the cytosol of neurons, circulating ovarian steroids combine with the corresponding hormone receptors. Binding steroids modify the conformation in these receptors, activating the transcription of genes (Farach-Carson and Davis, 2003; Nilsson et al., 2001). As a result, steroid signaling activates the production of specific proteins in neurons. The estrogen receptors have long been considered to be of only one kind (Walter et al., 1985). However, in 1996 Kuiper et al. cloned a novel estrogen receptor, resulting into the identification of two estrogen nuclear receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The existence of two different receptors presupposes that they play different roles in the regulation of several physiological and behavioral mechanisms. With regard to the involvement of estrogens in a large range of adaptive behaviors, a fine analysis of the actions of each receptor is crucial to the understanding of some psychiatric troubles and the development of treatment possibilities.

1.2 Rat sexual behavior

Just like humans, rats are very cosmopolitan. Their habitat range covers all continents, under every climate, from urban to rural areas. The rats frequently construct burrow systems as places of harborage (Calhoun, 1962), around which they wander in a rather large area described as their home range (Davis et al., 1948). It is therefore difficult to describe their specific habitat type. On the contrary, the social aspect of rat's life is much more stereotyped. Wild rats live in multi-male, multi-female groups, with a smaller proportion of male members than female ones (Calhoun, 1962; Leslie et al., 1952). Throughout the day, the rat engages in a number of solitary activities, for example foraging and scavenging in unfamiliar territory. Nevertheless, social interactions represent a substantial amount of its time-budget.

1.2.1 Male sexual behavior

Male sexual behavior is composed of a highly stereotyped sequence of copulatory acts including mount, intromission and ejaculation. Mounting behavior is characterized as a rat standing on its hind legs placing its forepaws on another rat's rump and displaying pelvic thrusts. The behavior, even though typical of male-female sexual interaction, is not limited to heterosexual encounters, and can be observed in male pairs or female pairs. Intromission is a mount accompanied by penile insertion into the vagina, followed by genital grooming. Finally, ejaculation shows longer penile insertion than intromission, accompanied with abdominal contractions and followed by an open arms posture (Lucio et al., 1994). In addition to copulatory acts, several male behaviors can be used to assess male interest into females. The most obvious indicator of male sexual arousal is the pursuit of the sexual partner. This is often associated with sniffing the anogenital region of the mate, even though this latter behavior is not always associated with sexual interaction itself.

Male sexual behavior has primarily been observed in standard copulation cages, in which a pair of rats is observed for a short period of time. The behavior is usually analyzed based on the observation of copulatory series. One series follows what is considered an increasing curve in sexual behavior intensity, starting with a first mount and ending with an ejaculation. However, in longer tests, a state of sexual exhaustion can be achieved, defined by the absence of copulatory acts within 30 min following the last ejaculation (Ågmo, 1999). Therefore, in more naturalistic settings, male behavior is expressed slightly differently. For example, in a seminatural environment a long period of male sexual inactivity can be achieved after any copulatory act, not necessarily after ejaculation (Chu and Ågmo, 2015a). Between these periods of sexual inactivity, the distribution of male sexual behavior is relatively stable (Chu and Ågmo, 2015a). Behavioral structure, even in the

case of relatively stereotyped behavior patterns, can be substantially modified depending on the observation context.

1.2.2 Female sexual behavior

Female most typical sexual behavior is lordosis posture. This posture involves arching the back and extending the neck while disposing the tail to the side to expose genital area. Female rats are normally considered to be in behavioral estrus, the period of sexual receptivity, whenever they demonstrate lordosis. Lordosis is expressed by receptive females in response to tactile stimulation of the back by male mount (Kow and Pfaff, 1973; McClintock and Adler, 1978; Pfaff, 1980).

The ventromedial nucleus of the hypothalamus (VMN) has been identified as the structure responsible for lordosis activation (Pfaff and Sakuma, 1979). Proteins produced through steroid signaling modulate the nerve signals from VMN down to the spinal cord, which results in the contraction of deep back muscles responsible for the lordosis reflex (Fig. 3). Thus, ovariectomy, reduction in the number of ERs, or lesion of certain brain areas, notably of the VMN, can alter or suppress the lordosis reflex.

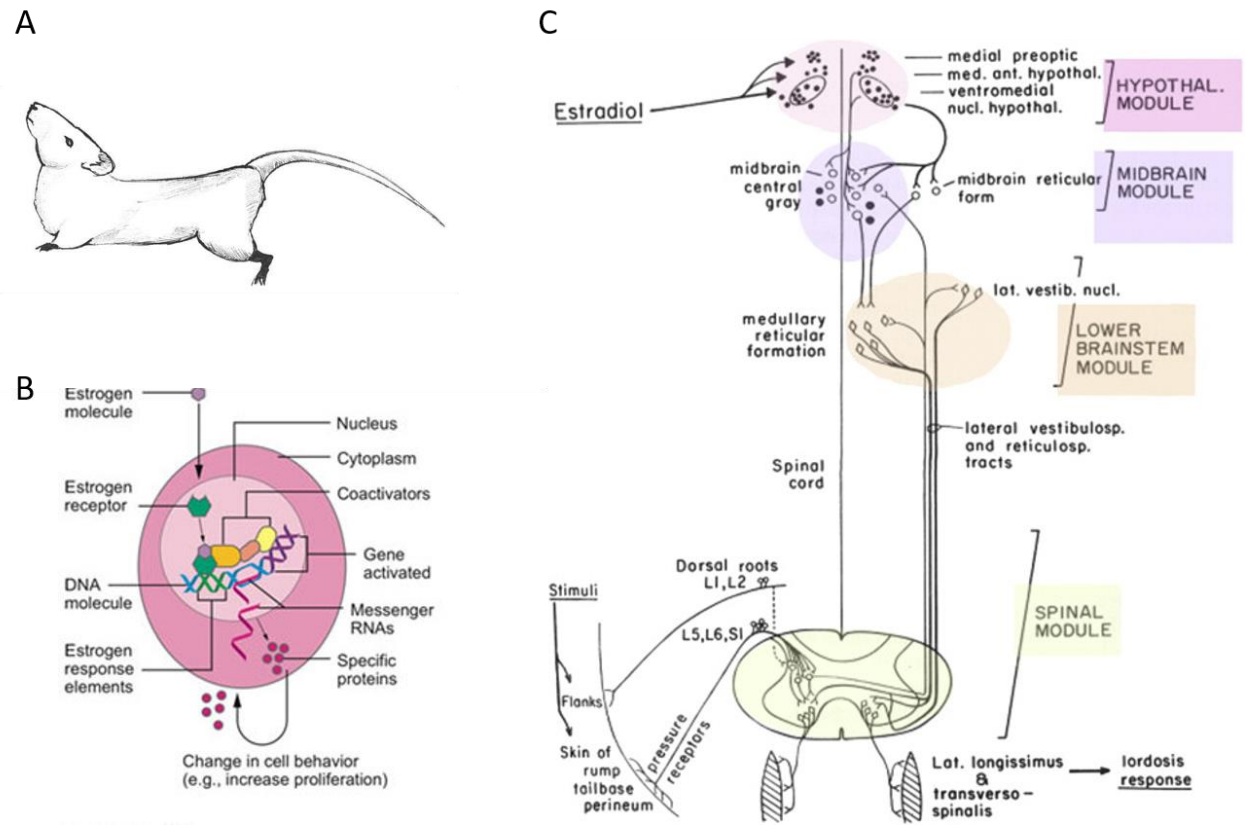

Fig. 3. A. Lordosis reflex. B. Diagram of hormone and hormone receptor (e.g. estrogen) showing receptor-mediated transcriptional activation. C. Neural circuitry mediating lordosis triggered by cutaneous stimuli and facilitated by estrogens action. Adapted with permission from Pfaff et al., 2000.

Among rats tested in pairs in standard copulation cages, females cannot escape the interaction. This results in females engaging in forced copulation, effectively blurring the lines of receptivity (Chu and Ågmo, 2016; Madlafousek and Hliňák, 1977). However, in naturalistic settings, females only engage in copulatory acts during behavioral estrus (Chu and Ågmo, 2015b). When females can experience paced mating, in seminatural environments or pacing chambers, the aversive properties of mating are reduced, and mating can induce positive affect (Paredes and Vazquez, 1999). Besides, when several male and female sexual partners are available, rats copulate

in promiscuous patterns: females copulate with multiple males during the entire period of estrus (Chu and Ågmo, 2014). Similar behaviors have been identified in humans, who have been shown to copulate in a completely random pattern, when the possibility is given to them (Friedman et al., 2008; Meunier, 2014; Tewksbury, 2002).

Receptive females display a number of behaviors reflecting the excitatory state, including rapid sequences of approach toward, orientation to, and withdrawal from proximity to a sexually active male (McClintock and Adler, 1978). Notably, ear wiggling, running, hopping and darting co-occur with lordosis during behavioral estrus. These behaviors, initially and still often labelled as "proceptive" (Beach, 1976), are not expressed in the absence of circulating steroids (Pfaff, 1980). More recently, it has been suggested that these behaviors be re-labeled as "paracopulatory", as they occur during copulation but their specific function remains unproven (Blaustein et al., 2009; Blaustein and Erskine, 2002). These behaviors can be activated by tactile stimulation from males (Ågmo, 2007; Ågmo et al., 2004) but also occur in the absence of any tactile stimulation. They are considered an indicator of female sexual motivation (Bergheim et al., 2015), and might enhance male motivation.

1.2.3 Implication of ERs in female sexual behavior

All female sexual behaviors are dependent on the ERα. These behaviors range from the display of paracopulatory behaviors and lordosis (Ogawa et al., 1998; Rissman et al., 1997), to being attractive for males and being attracted to males (Kavaliers et al., 2004; Kavaliers et al., 2004). Contrarily to the ERα, the ERβ does not contribute to female sexual behavior. Female mice lacking ERβ display normal sexual behaviors (Antal et al., 2012; Ogawa et al., 1999; Walf et al., 2008) and these behaviors are not restored in ovariectomized female rats treated with an ERβ agonist (Mazzucco et al., 2008).

1.3 Rat social behavior

1.3.1 Female pro- and anti-social behaviors

Prosocial behaviors, including approach patterns and interactions promoting social cohesion (e.g. olfactory investigation, allogrooming, huddling) (Barnett, 1963) are modulated by estrogens, notably due to estradiol effects on the serotonin and oxytocin systems (Bethea et al., 2002). Treatment with estradiol promoted pro-social, affiliative behaviors in rhesus monkeys (Michopoulos et al., 2011) and female rats (Walf and Frye, 2008). Rats exhibit a number of agonistic, antisocial behaviors, such as avoidance patterns, attacks and defensive behaviors. These behaviors can also be facilitated or inhibited as a result from estrogen actions (Albert et al., 1992). However, the role of estradiol on aggression has mainly been studied in the case of maternal behavior. Maternal aggression is disrupted in ovariectomized females and restored by estradiol treatment (e.g. Mayer and Rosenblatt, 1987). Estrogens also seemed to influence territorial aggression in non-pregnant, non-maternal rats, by suppressing aggression in estrus females (Barfield, 1984). Nevertheless, little is known about the effects of estradiol on pro-social and anti-social behaviors in nulliparous or cycling females.

1.3.2 Implication of ERs in female pro- and anti-social behaviors

Disruption of ERα gene in female mice enhanced offensive attacks toward same sex intruder compared to wild-type (Ogawa et al., 1996). Virgin ERα knock-out female mice showed increased aggression towards ovariectomized, hormone-primed same-sex intruders, a behavior reduced by estrogen treatment (Ogawa et al., 1998). From these results, ERα seems to down-modulate aggression. The effects of ERβ on aggression are still difficult to understand. Intact ERβ knock-out female mice did not show aggression towards female intruders (Ogawa et al., 1999). However, single acute administration of an ERβ agonist to adult ovariectomized female mice

reduced dominance score and increased the number of agonistic behaviors received during their interactions with a familiar, ovariectomized same-sex cage mate (Clipperton-Allen et al., 2008). Few studies have focused on describing estrogen actions on pro- and anti-social behaviors, and an extensive, transferable analysis of estrogen effects on social interaction is lacking.

1.4 Rat exploratory and fear behaviors

1.4.1 Exploratory behavior

Rats spend a substantial amount of time exploring their environment, in what seems to be a constant search for novelty (Barnett, 1963). Exploratory behaviors include approach behaviors, as well as behaviors of olfactory and visual exploration such as sniffing or rearing. Exploration of new territories can be measured by frequency and range of locomotion. Novelty-induced behaviors such as rearing or increased locomotion are mostly expressed in safe, familiar contexts (Oloruntobi et al., 2014). Consequently, a reduction in rat exploratory tendency is usually considered an indicator of fear or anxiety.

1.4.2 Fear- and anxiety-related behaviors

A number of classical tests are usually implemented to assess fear and anxiety levels. The most commonly used are the open-field test, the Vogel test, the light-dark compartment test, and the elevated plus-maze (Harro, 2018). Some behavioral indices can be observed and broadly compared among all these procedures. Briefly, an increase in latencies to enter the center of the open area or the light compartment is interpreted as increased anxiety, while increased total time spent and frequency of head dipping into these parts show decreased anxiety levels. Anxiety in these procedures also reduces locomotor activity, increases freezing and stimulates the display of stretch-attend posture and risk assessment. Fear is expressed through avoidance behaviors (escape and hide) and with other behaviors such as freezing or the startle reflex.

One of the crucial brain areas for fear response is the amygdala, notably the central nucleus of the amygdala (CeA), which has the capacity to modify the HPA axis (Herman and Cullinan, 1997). Most research on stress and psychiatric diseases has focused on this structure (Lebow and Chen, 2016), which regulates rapid-onset, short-duration behaviors occurring in response to specific threats (Davis and Shi, 1999; Duvarci et al., 2009; Walker et al., 2003; Walker and Davis, 1997). Even though most of the literature agrees that the CeA is mainly involved in immediate fear responses, a review of the role of amygdala in these responses in rats suggested that immediate reactions are mediated by the medial portion of the CeA, while its lateral portion could mediate more sustained responses (Davis et al., 2010).

1.4.3 Implication of ERs in fear and anxiety responses

Contradictory results were obtained regarding the role of ERα in fear and anxiety responses. One study reported that ERα knock-out mice were not different from wildtype in several of the anxiety procedures (Krężel et al., 2001). However, it has also been reported that a selective ERα agonist had anxiogenic effects in fear-inducing environments (elevated plus-maze and novel open field) (Lund et al., 2005). It has also been found that the ERα is anxiogenic in the light/dark box and in a brightly lit open field (Spiteri et al., 2012, 2010a).

In parallel, activation of ERβ has been consistently reported to have anxiolytic effects. Treatment with an ERβ agonist reduced indicators of fear in an elevated plus-maze in female rats (Kudwa et al., 2014) and female mice (Krężel et al., 2001; Oyola et al., 2012; Alicia A. Walf et al., 2008), whereas ERα had no effect. Therefore, it seems that ERα and ERβ agonists have opposite effects in novel/fear-inducing contexts. This makes little sense biologically, since both receptors are activated at the same time by estradiol. However, the distribution of ERs in the brain is heterogenous. Differential activation of brain structures might shed light on this contradiction.

1.5 On the induction and measurement of emotions

1.5.1 Behavior as a tool for interpreting emotion

From an evolutionary standpoint, emotions seem to have appeared to equip organisms with abilities that allow them to seek valuable resources/ rewards and avoid harm/ punishment (Ikemoto and Panksepp, 1994; Rolls, 2000). There is a large amount of evidence in scientific literature that points out to the existence and critical importance of emotions in terms of survival, both in humans and non-humans animals (Berridge, 1996; Berridge and Robinson, 2003; Davis and Whalen, 2001). Nowadays, emotions are regarded as a multicomponent system, comprising psychic, behavioral and physiological responses, and, in humans, subjective consciousness (Paul et al., 2005; Shuman et al., 2017).

1.5.2 Assessing animal emotions in scientific research

Animal emotions research has mostly focused on the measurement of negative affective states, as they appear easier to identify (Paul et al., 2005). Even considering the increasing public concern for animal welfare, and the social imperative for personal development and happiness, positive affects are rarely addressed and research remains biased toward the study of negative experiences (Webb et al., 2018). This bias leaves out a large panel of emotional effects, not only offering an incomplete view of the issue of mental health, but also distorting the prism of data interpretation.

Interestingly, while behavioral studies extensively describe “anxiety-related behaviors”, there is no mention of behaviors typical of a positive emotional state. Reports of “hedonic behaviors” refer to active reward-seeking (e.g. Duncko et al., 2003; Grippo et al., 2006) and are therefore irrelevant to the expression of positive affects. With animals, the lack of self-reports calls for the use of physiological and behavioral indices. Physiological indicators include changes in the

autonomic function, activity of the hypothalamic-pituitary-adrenal axis, and measures of neuroendocrine activity. According to Webb and collaborators (Webb et al., 2018), behavioral indicators can indicate momentary affect (e.g., spontaneous postures and behaviors, facial expressions, vocalizations, approach or avoidance responses to novel stimuli) and cognitive biases linked to particular affective states (judgment, attention, and memory). Identifying behavioral characteristics of different affects requires a comparison in behavior patterns in a range of different contexts.

1.6 Manifest for the use of externally valid procedures

A recent review of anxiety studies in rodent models highlighted the challenge of anxiety measurements, and emphasized the need for clearer definitions of the measured variables and conditions used, in order to achieve greater transferability (Harro, 2018). Indeed, transferability of results obtained in clinical trials to real-life situations is not always granted. Brunswick defined procedures which results are generalizable to other contexts as procedures with an external validity (Brunswick, 1955; Brunswik and Kamiya, 1953). Such settings are particularly relevant in studies of behavioral responses to experimental manipulations and treatment (Peters et al., 2015). To the contrary, observation in simplified contexts are specific to this very context, and can difficultly be verified or repeated in different procedures. For this reason, the field of behavioral neuroscience would benefit from a greater use of externally valid procedures.

1.7 Goals of the thesis

Estrogens are strongly involved in the mobilization and regulation of socio-sexual behaviors. Both ERα and ERβ modulate behavioral responses in a different manner, and their activation produces different behavioral patterns depending on the brain area activated. Similarly, both receptors differently regulate fear responses and several studies so far pointed to their opposite

properties. However, in most studies the role of environmental context and emotional stimulations has been overlooked. In light of these considerations, the present thesis aims to unveil the differential role of ERs in a naturalistic environment with external validity, in adult female rats with altered ER expression.

Three different experiments were carried out in order to assess socio-sexual and fear-related behaviors in female rats hosted in a seminatural environment, when submitted to either positive or negative emotional stimulations. First, I characterized the behavior of female rats in estrus in the seminatural environment. Then, two different approaches were used, either systematically administering ER agonists to female rats, or silencing ERs in specific brain regions, specifically the VMN or the CeA. This made it possible to assess the effect of each ER on these behaviors, as well as the interaction of ERs' properties with the environmental context.

Detailed behavioral observation and analysis of rat behavior in a procedure with external validity can rarely be implemented. The studies presented in this thesis will hopefully give new insight into behavioral actions of ERs, while potentially contributing to an understanding of the etiology of some psychiatric disorders.

2. General methods

2.1 Externally valid procedure

2.1.1 Seminatural environment

Across the years, several studies have observed rodent behavior in seminatural environments (Blanchard et al., 1985; McClintock et al., 1982; McClintock and Adler, 1978; McClintock and Anisko, 1982). In accordance with these previous studies, and with key elements of the rat's physical and social environment, it was possible to build a seminatural environment incorporating or mimicking most of these elements. The environment described here has been used previously in a number of studies (e.g. Chu and Ågmo, 2015a, 2015b, 2014) and has been used in all the experiments presented in this thesis. The seminatural environment consisted of two parts, a burrow system (120 × 210 cm) and an open area (120 × 210 cm), connected by 4 small opening doors (8 × 8 cm) (Fig. 4). Overall size of the seminatural environment was 2.1 × 2.4 m. The open area and the burrow were separated by a completely opaque black fabric, preventing any light to enter the burrow system. Both the burrow and the open area were divided into zones, making it possible to quantify rats' locomotor activity by assessing how often they transited from one zone to another (Fig. 4B). Two infrared lamps (850 nm; model Sal60, New Surway Digital Technology, Shenzhen, Guangdong, P.R. China) were installed in the ceiling, providing light for video recording. The open area was submitted to a reverse 12L:12D light cycle, light being on from 11 pm to 11 am. This produced a light intensity of 30 lx during night phases and 180 lx during day phases, as measured on the floor. Dawn and dusk were simulated by 30 min light transitions. The humidity level in the seminatural environment was 55±10% and the temperature 21±1°C. The sound level approximated 40 dB, due to the ventilation system providing 15 air changes per hour.

Two nozzles, one in the back tunnel of the burrow, one in the far wall of the open area, were connected to an odor distribution system (Olfactory Stimulus Package, Medical associates, Georgia, Vt) producing an airflow of 3 l/min. This airflow could be directed through one of two jars containing odorants or made to bypass the jars and consist of unscented room air instead. Finally, a sound system composed of two A60 stereo speakers from Creative (Clas Ohlson, Norway) could be used to produce auditory stimulation. The entire seminatural environment was filmed with 2 cameras, one in the burrow part and one in the open area, using The Media Recorder 2.5 (Noldus, Wageningen, The Netherlands).

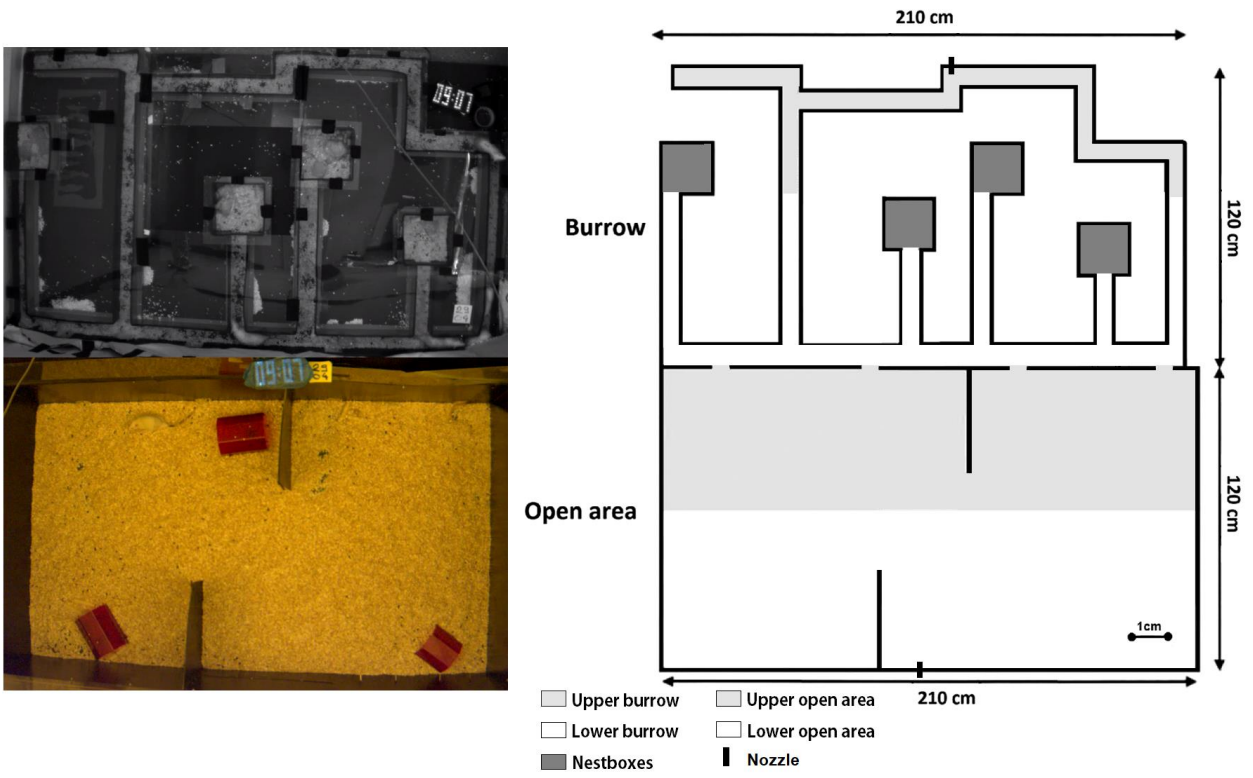

Fig. 4. A. Picture of the seminatural environment. B. The division in zones.

2.1.2 Emotion-inducing stimuli

Once the rats are established in the seminatural environment, this environment becomes stable and familiar, and, to a certain extent, safe. At this point, rat behavior can be considered at

baseline. In order to induce changes in rat behavior, I proposed emotional challenges based on the stimulation of senses that are well developed in rats: olfaction, gustation and audition. After thorough examination of the literature, I decided to implement five different stimuli. Lavender essential oil (*Lavandula angustifolia*) which has anxiolytic properties in rats and humans (Bradley et al., 2009; Shaw et al., 2007); and fox odor (2,5-dihydro-2,4,5-trimethylthiazoline, TMT), a predator odor aversive to the rats (Endres et al., 2005; Endres and Fendt, 2009; Fendt et al., 2005) were chosen as olfactory stimuli. For auditory stimulation, Mozart Sonata for two pianos (K448) has been reported to have anxiolytic effects (Chikahisa et al., 2007; Escribano et al., 2014), and loud white noise is a common stressor know to produce fear reactions (Weyers et al., 1994). These two stimuli were played at 50-60 dB and 90 dB, respectively. Finally, I provided the rats with chocolate-flavored food in high quantities to avoid competition-related stress. Chocolate is a highly palatable food for rats (and me), and is consumed quickly (Boswell et al., 2006; Lampert et al., 2013; Reynaert et al., 2016).

The stimuli were presented in a fixed order: lavender odor, music, chocolate, white noise, and fox odor. A 50-minutes interval separated the end of each stimulus from the beginning of the following one, allowing for behavioral recovery.

2.1.3 Studies design

Each group of rats ran in the seminatural environment consisted of 7 sexually naïve rats, 3 males and 4 females. All animals were unknown to each other. The males were always left intact. The females underwent pharmacological or surgical treatments to modify ER expression. The animals were housed in the seminatural environment for 8 days. They were introduced on day 0 at 13:00. On day 7, the sequence of emotion-inducing stimuli was initiated, starting at 13:00 and finishing at 18:30.

2.2 Behavioral observations

2.2.1 Analysis of behavioral durations and frequencies

Video recording of the rats' behavioral activity in the seminatural environment allowed for scoring a large range of their behaviors. This was done with The Observer XT 12.5 (Noldus, Wageningen, The Netherlands).

2.2.2 Analysis of the behavioral flow

The richness of behavioral observation is constituted not only by the quantification of isolated behavioral items, but also by the chronological organization of behavioral patterns. I proposed to consider the behavioral flow as a speech, and to analyze it as so. The software Iramuteq (Interface de R pour les Analyses Multidimensionnelles de Textes et Questionnaires) made it possible to understand how behavior patterns were structured depending on a specific emotion-inducing stimulus and/or on individual treatments received.

3. Can emotion-inducing stimuli modify fear and anxiety-related behavior?

3.1 Introduction

Anxiety models rely on a number of behavioral indices that are observable in most standard procedures. However, in non-standard procedures such as seminatural environments, these responses might be expressed differently or simply not expressed. Notably, a classical indicator of fear (freezing occurrence) was found to be almost absent in a procedure where mice were provided with a shelter (Vale et al., 2017), a feature unavailable in the elevated plus-maze, for example. This observation is symptomatic of a general issue regarding the transferability of behavioral studies' results. I observed rats treated with either anxiogenic yohimbine or anxiolytic chlordiazepoxide under different emotional contexts to provide insight onto the variability of fear- and anxiety-related behavior in a seminatural environment.

3.2 Results of Paper I

3.2.1 Different emotion-inducing stimuli elicit different profiles of behavioral response

Exposure to lavender showed to stimulate sexual behaviors (lordosis and paracopulatory behaviors), behaviors of self-maintenance such as drinking, self-grooming and resting alone, and enhanced exploration of the open area. This last response is also observable in standard models of anxiety and considered an indicator of decreased anxiety. Taken together, these findings suggest an anxiolytic effect of lavender odor, or simply that lavender odor is attractive to the rats.

Exposure to chocolate increased both pro- and anti-social interactions. This increase can result from competition for chocolate access, and from social transmission of food-related information such as flavor, location and quantity. Consequently, it is difficult to formulate any conclusion other than that the rats ate chocolate with gluttony.

Music, white noise, and fox odor all appeared to be aversive to the rats. In particular, exposure to white noise inhibited sexual behaviors, behaviors of self-maintenance, and suppressed the exploration of the open area. White noise had evident aversive fearful properties that materialized through the behavioral modifications induced. Interestingly, exposure to white noise also stimulated locomotor activity in the burrow. Increased exploration and locomotion are usually considered indicators of low anxiety or novelty (Koss et al., 2004; Oloruntobi et al., 2014). However, in classical procedures, rats can rarely experience a spacious safe space such as that provided by the complex burrow system, where the rats gathered during exposure to white noise.

Exposure to fox odor showed aversive properties, as expected with regard to the notoriously anxiogenic effect of this predator odor in other procedures (Endres et al., 2005; Endres and Fendt, 2009; Wallace and Rosen, 2000). Interestingly, exposure to music seemed aversive to the rats while it was expected to have anxiolytic properties (Chikahisa et al., 2007; Escribano et al., 2014). Positive effects similar to that observed in humans have been found on physiology and behavior of laboratory animals (Alworth and Buerkle, 2013). However, several elements can help give meaning to my results. First of all, rats have an innate preference for silence over 40-, 20-, then 10-kHz tones (Soga et al., 2018) and when given the choice, they will spend more time in a silent room than in a room playing Mozart's K448 (Zhang et al., 2009). Therefore, with the seminatural environment being relatively quiet (~40 dB), music may have constituted a disturbance to the rats. Another interesting point is that most studies of behavioral effects of Mozart's music investigated rats' performance after music exposure. To the contrary, in my experiment, I always observed the rats during exposure to music. In humans, the positive effects of Mozart's music have been attributed to elevated mood and arousal (Cassity et al., 2007; Thompson et al., 2001) due to music enjoyment. Considering that silence is preferred over music by rats, it is possible that music per se is not

responsible for the observed effects, but that its termination and the increased arousal thus induced, is. This is consistent with findings of Paper III, in which I found increased locomotor, exploratory and sexual activity right after the offset of aversive white noise.

Different emotion-inducing stimuli induced different behavioral profiles. Except for music, all emotion-inducing stimuli elicited behaviors pattern consistent with the expected affect. It is reasonable to assume that these response profiles may result from different affective states in the rats.

3.2.2 Indicators of fear and anxiety in a seminatural environment

Anxiogenic yohimbine and anxiolytic chlordiazepoxide given to estrous female gave me insight into rat's behavior under different levels of anxiety. I found few effects of the drugs in Paper I. Yohimbine significantly increased lordosis quotient and self-grooming. It also decreased the latency to flee the noise, consistently with its anxiogenic properties. Females treated with yohimbine were associated with occurrences of rearing and with chocolate exploration.

Chlordiazepoxide significantly increased the frequency of hiding alone, a behavior specific to white noise exposure. In a co-occurrence analysis, chlordiazepoxide was not different from control females treated with saline when all emotion-inducing stimuli were collapsed.

3.2.3 Emotional regulation in an externally valid procedure

A study of expression and assessment of emotional responses in sheep suggested that increase in perceived controllability and increase in social support would decrease markers of anxiety (Greiveldinger, 2007). In the seminatural environment, rats pace freely, take shelter into the burrow, and actively engage or avoid social interactions. This provides a certain controllability

over the stimuli and allows the rats to employ their adaptive capacities and thus their ability to self-regulate in response to environmental changes (Koolhaas et al., 2011).

The hypothesis that social support attenuates fear responses has been formulated in the past (Davitz and Mason, 1955). This phenomenon, called social buffering, refers to lower expression of fear and anxiety in presence of a fearful stimulus when accompany by a conspecific (Kiyokawa and Hennessy, 2018). In the seminatural environment, rats interacted with six familiar conspecifics, in an established group hierarchy. It is likely that this social configuration consistently reduced fear and anxiety responses. This is also relevant to the higher frequency of hiding alone observed in females treated with chlordiazepoxide. These female might have exhibited a lower need for social buffering, which would be consistent with chlordiazepoxide's anxiolytic properties.

3.3 Conclusions

The five emotion-stimuli implemented in the experiment elicited different patterns of behavioral responses. Overall, chlordiazepoxide- and yohimbine-treated females showed profiles of response consistent with anxiolytic and anxiogenic effect, respectively.

In response to uncertainties in transferability of behavioral studies and interpretation reliability, and to the call for ethological concepts to enhance the translational value of animal models (Peters et al., 2015), the use of the seminatural environment highlighted the effect of social buffering and controllability as compensatory mechanisms in response to environmental variations.

Paper I

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

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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

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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

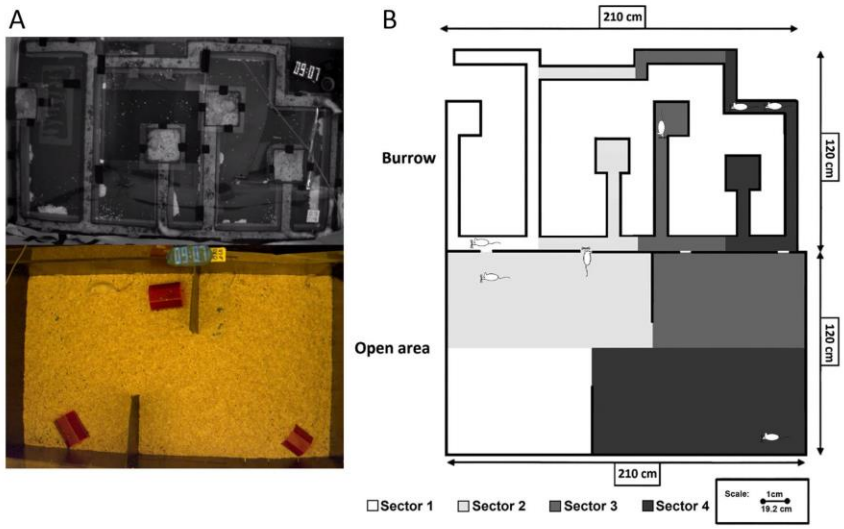

Fig. 1. A. Picture of the seminatural environment. **B**. The division in zones.

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 seminatural 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

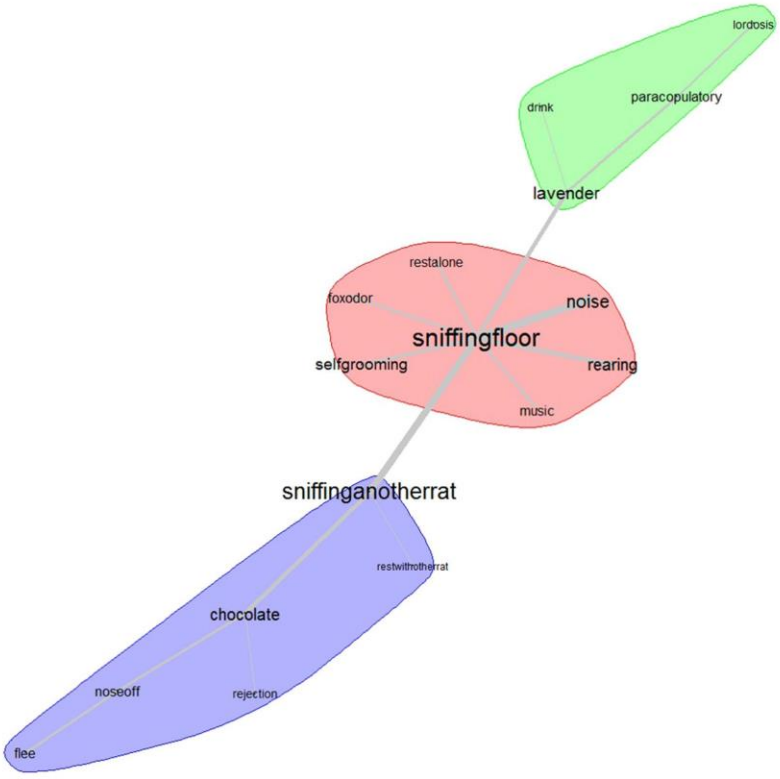

Fig. 2. Co-occurrence analysis showing main behavioral associations typical of each of the experimental conditions over the course of the entire sequence of emotion-inducing events (excluding event-specific behaviors). Clusters of behavioral association are represented in halos of different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

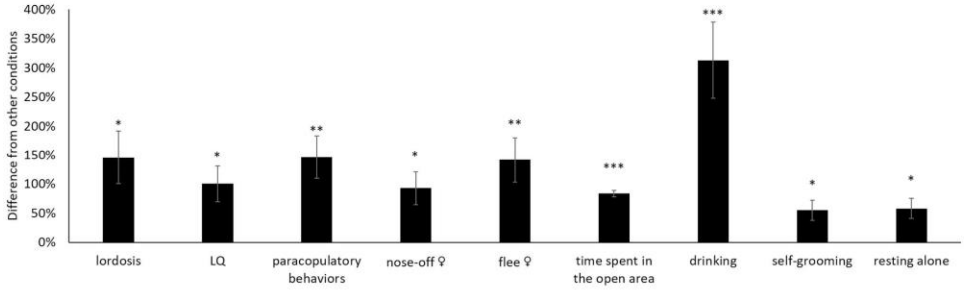

Fig. 3. Effect of exposure to lavender odor on female behavior. Only behaviors significantly altered by the exposure to lavender compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.

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

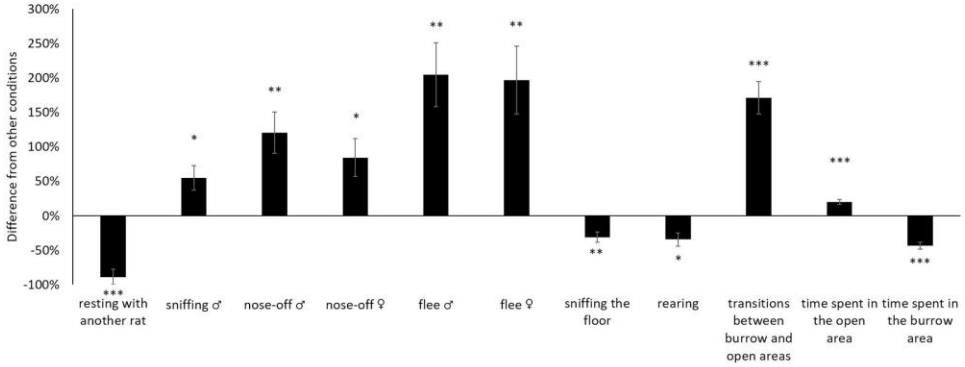

Fig. 4. Effect of exposure to chocolate pellets on female behavior. Only behaviors significantly altered by the exposure to chocolate compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.

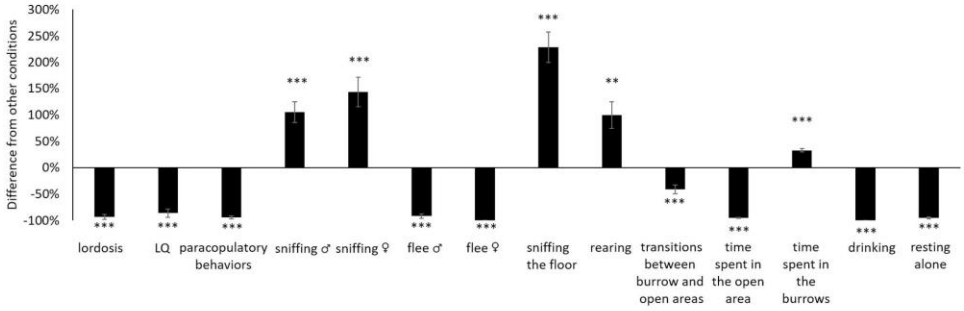

Fig. 5. Effect of exposure to white noise on female behavior. Only behaviors significantly altered by the exposure to white noise compared to the mean of the other conditions are shown. Data are mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001. N = 32.

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

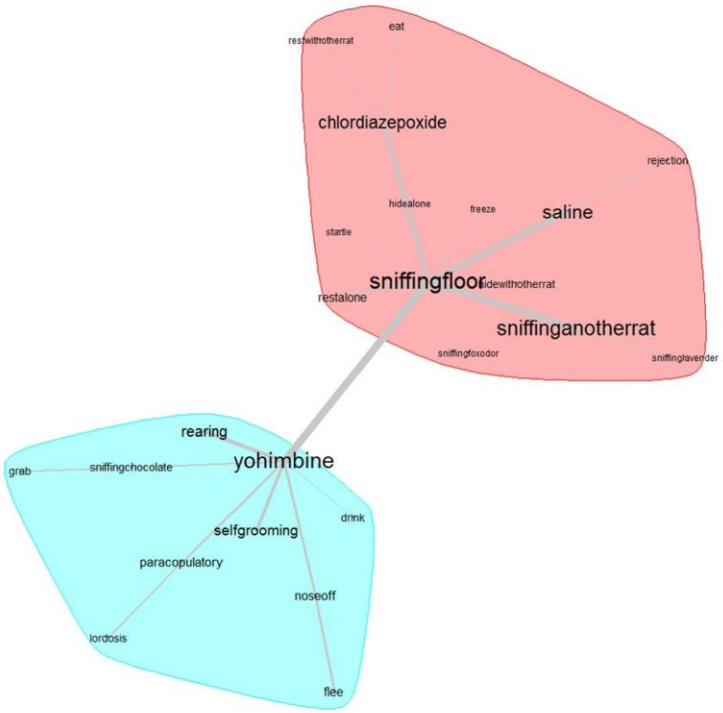

Fig. 6. Co-occurrence analysis showing main behavioral associations typical of each of the treatments, over the course of the entire sequence of emotional challenges (including event-specific behaviors). Clusters of behavioral association are represented in halos of different colors. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

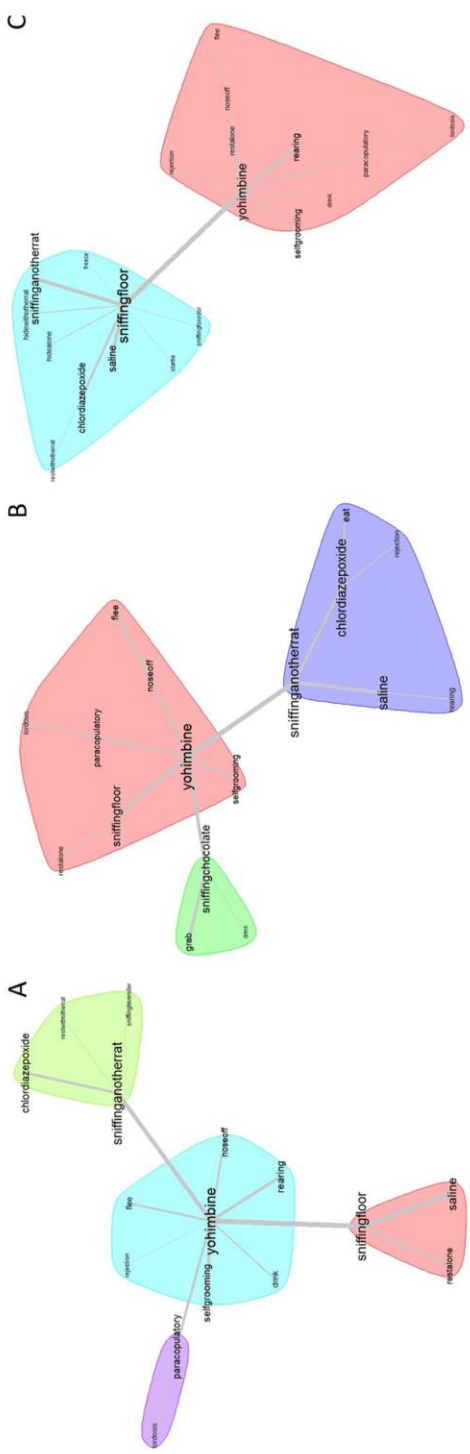

Fig. 7. Co-occurrence analysis showing main behavioral associations typical of each of the emotion-inducing event. **A.** Lavender odor, **B.** Chocolate, **C.** Aversive conditions (music, white noise and fox odor). Clusters of behavioral association are represented in halos of different colors. (For 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, antisocial 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 [$\chi^2_2 = 1.130$, $P = 0.568$] (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.

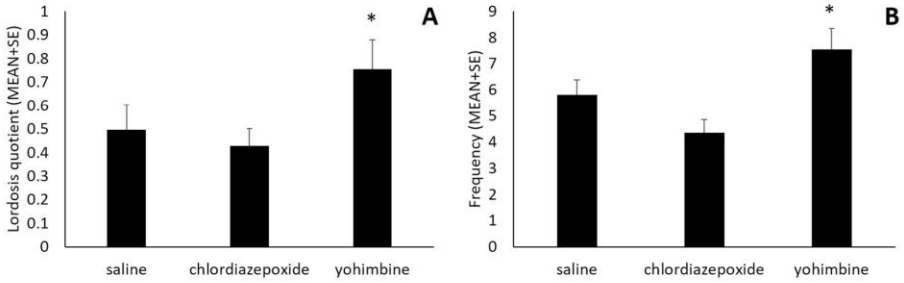

Fig. 8. A. Effect of the treatment on lordosis quotient (all experimental condition collapsed). **B**. Effect of the treatment on self-grooming (all experimental condition collapsed). Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: n = 10, chlordiazepoxide: n = 11, yohimbine: n = 11.

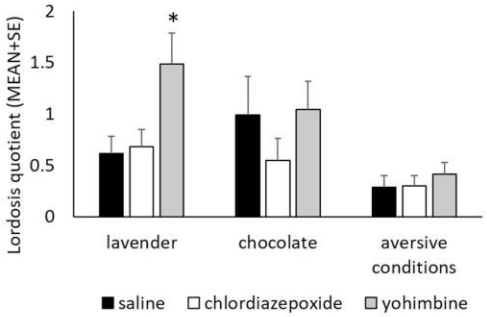

Fig. 9. Effect of the interaction between the treatment and the experimental condition on the lordosis quotient. Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: n = 10, chlordiazepoxide: n = 11, yohimbine: n = 11.

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.

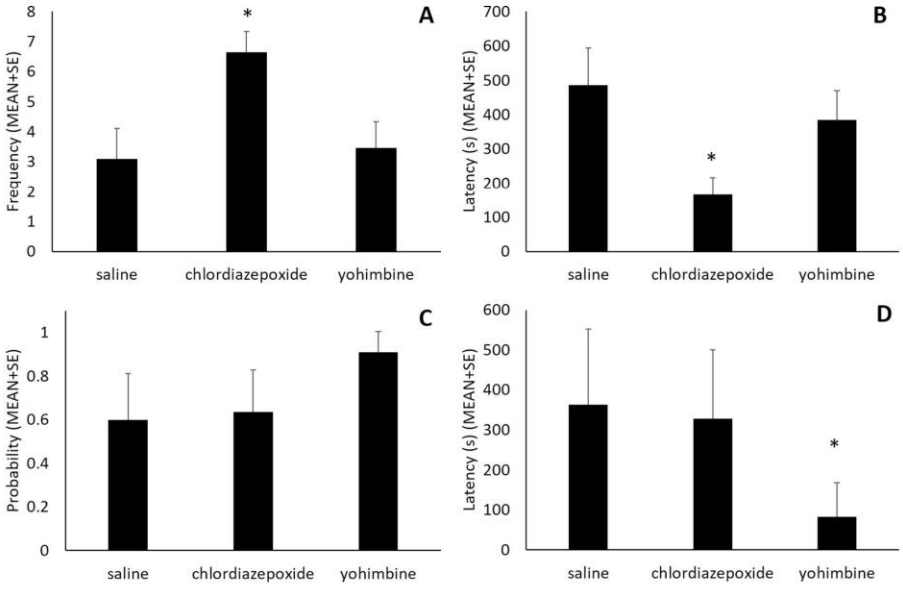

Fig. 10. Effect of the treatment on white noise-specific behaviors. **A.** Frequency to hide alone. **B.** Latency to hide alone. **C.** Probability to flee the noise at its onset. **D.** Latency to flee the noise at its onset. Data are mean ± SEM. *, P < 0.05, different from the saline group. Saline: 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 therapeutical 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 yohimibine 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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4. Systematic administration of ER agonists

4.1 Introduction

In order to characterize the differential roles of ERα and ERβ in a procedure with external validity, I used well-established ER agonists to reveal the role of each agonist in socio-sexual and fear-related behaviors. I treated female rats with propyl-pyrazole triol (PPT; ERα agonist) or diarylpropionitrile (DPN; ERβ agonist). As the implication of ERα in female sexual behavior and attractivity had already been known through standard procedures (Mazzucco et al., 2008), it was of interest to see how these behaviors were modulated in different emotional contexts. In addition, ERβ agonist previously showed anxiolytic properties (Lund et al., 2005; Oyola et al., 2012) in procedures such as the elevated plus-maze, the open-field, and the light-dark exploration tests. Whether these anxiolytic properties would express differently in safe or aversive contexts remained uncertain. Thus, I sequentially treated female rats with PPT + progesterone, DPN + progesterone, or estradiol + progesterone, and compared them to a control (oil + progesterone).

4.2 Results of Paper II

4.2.1 Confirmation of emotion-inducing stimuli effect

Independently from the treatment given, females responded to the emotion-inducing stimuli in a similar way as found in Paper I. Exposure to lavender odor stimulated locomotor activity in the open area compared to this behavior at baseline. Exposure to music reduced the time spent in the open area. Exposure to chocolate increased sniffing of the seminatural environment and male sniffing of the females. White noise inhibited female sexual behavior and exploration of the open area, while increasing locomotor activity in the burrow. Exposure to fox odor reduced male pursuit of the females.

4.2.2 Role of ERα and ERβ agonists

The main effect of ERα agonist PPT was its significant activation of lordosis and paracopulatory behaviors compared to ovariectomized females treated with oil. It maintained sexual behaviors at levels somewhat similar to that of estradiol benzoate, whereas DPN failed to activate lordosis posture. In addition, females treated with PPT received male mounts and were pursued by the males, while females treated with DPN were not more attractive to the males than females treated with oil.

The proportion of females fleeing the noise at its onset was higher in the PPT-treated group. In the co-occurrence analysis, PPT-treated females were often associated with exploratory behaviors and chocolate-related behaviors. This was consistent with the arousing properties of estradiol, heightening fear in threatening contexts and increasing activity in safe environments (Morgan et al., 2004).

Administration of DPN did not modify the frequency of any of the recorded behaviors. However, DPN-treated females appeared in a different cluster than females treated with oil only during aversive stimuli, music, white noise and fox odor. These females were then associated with self-maintenance behaviors (drinking in the open area, self-grooming) as well as with hiding alone or with another rat during white noise. This might indicate a different way of DPN-treated females to cope with aversive situations. In particular, in Paper I, I found that exposure to aversive white noise suppressed occurrences of self-maintenance behaviors. The association of DPN-treated females with these behaviors during exposure to aversive stimuli proposes that this agonist was anxiolytic.

4.3 Conclusions

The study confirmed the necessity of ERα for sexual receptivity regardless of the environmental context. In addition, ERα showed arousing properties resulting in contrasted behavioral responses depending on the emotional stimulus induced. ERβ agonist modified the structure of behavior only during aversive stimuli, suggesting anxiolytic properties and confirming findings of classical anxiety models.

Paper II

Behavioral responses to emotional challenges in female rats living in a seminatural environment: The role of estrogen receptors

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ABSTRACT

Estrogen receptors (ERs) are involved in sexual as well as non-sexual behaviors. In the present study we assessed the effects of stimuli inducing positive or negative affect on sociosexual, exploratory and fear-related behaviors of female rats housed in groups (4 females, 3 males) in a seminatural environment. Ovariectomized females were treated with oil, 17β-estradiol benzoate (EB, 18 μg/kg), the ERα agonist propylpyrazoletriol (PPT), or the ERβ agonist diarylpropionitrile (DPN) (both 2 × 10 mg/rat). On the test day, the females were exposed to a sequence of events consisting of lavender odor, Mozart's Sonata for Two Pianos K448, chocolate pellets, white noise and fox odor (2,3,5-Trimethyl-3-thiazoline, TMT). All these events are known to induce positive or negative affect. Behavior was carefully observed from the video record. White noise suppressed sexual behaviors and reduced the time spent in the open area of the environment. TMT had no consistent effect whereas exposure to music caused avoidance of the open area. Exposure to chocolate increased exploratory and social behavior. Lavender odor enhanced exploratory behavior. PPT and EB stimulated sexual behaviors, whereas DPN was ineffective. Co-occurrence analyses of the sequence of behavioral patterns revealed that PPT and EB consistently belonged to clusters different from oil and DPN, whereas DPN was separate from oil only under fear-inducing experimental conditions. These data, from a procedure with external validity, confirm that the ERα is crucial for sexual behaviors, that these behaviors are reduced under stressful conditions, and that the ERβ may have some role in fear-related behaviors.

1. Introduction

Female rodents only express sexual behavior when their brain is exposed to appropriate concentrations of ovarian hormones. Estrogens and progesterone normally act synergistically, but high doses of estrogens can activate all aspects of female sexual behavior in the absence of progesterone, whereas progesterone is ineffective in the absence of estrogens regardless of dose (e.g. Södersten and Eneroth, 1982). It is known that all female sexual behaviors, including being attractive for males and being attracted to males (reviewed in Le Moëne and Ågmo, 2018) as well as the display of paracopulatory behaviors and lordosis (e.g. Ogawa et al., 1998; Rissman et al., 1997), are dependent on the estrogen receptor α (ERα). The estrogen receptor β (ERβ) does not contribute to these behaviors, since female mice lacking this receptor show perfectly normal sexual behaviors (Ogawa et al., 1999; Walf et al., 2008a, 2008b; Antal et al., 2012) and since ERβ agonists are unable to activate these behaviors in ovariectomized female rats (Mazzucco et al., 2008).

In addition to being necessary for the display of sexual behaviors, estrogens have several behavioral effects, some of which may be relevant for the sex behaviors. Among those, modifications of fear and anxiety responses might be particularly important. There are also data suggesting that estrogens may alter the response to events inducing positive affect, for example the ingestion of tasty foods like sucrose or chocolate (e.g. Clarke and Ossenkopp, 1998; Reynaert et al., 2016). However, the effects of fear-inducing stimuli or situations or of stimuli causing positive affect on sexual behavior in female rats have not been studied. In fact, available data are limited to studies of the effects of stress on subsequent sexual behavior. It can be argued that fear stimuli activate stress responses, and studies of stress could therefore provide some information about the potential effects of fear on sexual behavior. Acute stress in the form of short restraint reduces the display of female copulatory behavior in females rendered sexually receptive with estradiol alone, whereas no effect was observed in females given estradiol + progesterone (Truitt et al., 2003). When females can pace sexual interaction, restraint reduces the time spent with the male as well as the number of mounts received regardless of the presence or absence of progesterone. Receptivity was not modified, though (Uphouse et al.,

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2005). Interestingly, restraint stress had no effect in a test for sexual incentive motivation (Uphouse et al., 2008). It appears, then, that acute stress has minor or no consequences for female sexual behavior. Chronic stress, however, has consistently been found to facilitate the display of lordosis and paracopulatory behavior and to reduce rejections (Brotto et al., 1999; Williams et al., 1992).

A common feature of all these studies is that they have evaluated the effects of prior, but not present, stress. Thus, the immediate consequences of fear-inducing stimuli for sexual behavior remain unknown. There is, however, abundant evidence for a role of estrogens in non-sexual, anxiety-like responses. Treatment of ovariectomized mice and rats with estradiol has been reported to enhance the time spent on the open arms of an elevated plus maze (e.g. Nomikos and Spyraki, 1988), or in the center of an open field (e.g. Walf and Frye, 2007), and to reduce a passive avoidance response (Díaz-Véliz et al., 1997). All these effects are usually interpreted as suggesting reduced fear or anxiety. Other studies have failed to find an effect of estradiol in anxiety tests (e.g. Walf and Frye, 2008; Martínez-Mota et al., 2000), and still others found anxiogenic effects (Mora et al., 1996). One hypothesis proposed to account for these contradictory observations is that estrogens are anxiogenic in threatening environments and anxiolytic in safe environments (Morgan and Pfaff, 2001). Such an effect could conceivably be useful for assuring that another ERα dependent behavior, copulation, occurs more easily in safe than in dangerous environments (see, e.g. Frye et al., 2006, for an elaboration of this argument). Direct experimental evidence for this conjecture is lacking, though.

The anxiolytic-like effects of estrogens are often attributed to actions at the ERβ. Mice without a functional ERβ are more fearful than the wildtype (Krezel et al., 2001), and treatment with a selective ERβ agonist reduces fear in female rats (Kudwa et al., 2014) and mice (Krezel et al., 2001; Oyola et al., 2012; Walf et al., 2008b), whereas selective ERα agonists are ineffective. It has also been reported that ERα knock-out mice are not different from the wildtype in several of the anxiety procedures (Krezel et al., 2001). However, anxiogenic effects of a selective ERα agonist in fear-inducing environments (elevated plus maze and novel open field) have been reported (Lund et al., 2005). It has also been found that the ERα is anxiogenic in the light/dark box and in a brightly lit open field (Spiteri et al., 2010b; Spiteri et al., 2012). Thus, it can be proposed that ERα and ERβ agonists might have opposite effects in fear-inducing contexts.

One of the purposes of the present study was to determine whether fear-inducing stimuli actually inhibit female sexual behavior, and if agonists selective for the ERα and ERβ would have different effects on the nonsexual responses to these stimuli. To that end, ovariectomized females were given either estradiol or selective ER agonists. Fear was induced by exposing the females to a 90 dB white noise or to synthetic fox odor. Loud noise as well as 2,5-dihydro-2,4,5-trimethylthiazoline (TMT) produce strong fear responses in rats (e.g. Endres et al., 2005; Fendt et al., 2005; Homiack et al., 2017; Weyers et al., 1994).

Another purpose of this study was to evaluate the effects of stimuli inducing positive affect rather than fear, and the potential role of the ERs for responses to such stimuli. Estrogen-modulation of responses to attractive, non-sexual stimuli has only been systematically studied with regard to food ingestion. It is well known that estrogens reduce food intake (e.g. Butera, 2010). It appears that the ERα is responsible for the effects of estrogens, since ERα knockout mice do not reduce food intake in response to hormone treatment (Geary et al., 2001). Moreover, a specific ERα agonist does reduce food intake whereas an ERβ agonist is ineffective (Shen et al., 2017). It has been suggested that post-ingestive factors rather than reduced hedonic impact of tastants underlie the reduced food intake (Hrupka et al., 1997; Flanagan-Cato et al., 2001). This proposal is reinforced by the fact that estrogens enhance the hedonic response to and consumption of tasty foods, such as sucrose (Clarke and Ossenkopp, 1998) or chocolate (Reynaert et al., 2016; Boswell et al., 2006; Lampert et al., 2013).

A less known effect of estrogens is to increase the fear-reducing effect of music. It has repeatedly been reported that music has anxiolytic activity in several of the standard procedures (Li et al., 2010; Cruz et al., 2015). When the sonata for 2 pianos (Köchel number 448) by W.A. Mozart is played to ovariectomized female rats, the anxiolytic effect is reduced compared to that found in proestrus females, but it can be restored by treatment with estradiol (Escribano et al., 2014). In the same study, it was found that the anxiogenic effect of loud white noise was not altered by ovariectomy or estrogen treatment. In mice, the effects of music seem less dependent on ovarian hormones, although both estradiol and progesterone had some effects (Chikahisa et al., 2007). The potential role of the different ERs has not been explored.

Another stimulus with anxiolytic effects in several rodent procedures is the odor of lavender oil (Umezu et al., 2006; Shaw et al., 2007; Tsang and Ho, 2010; Tsang et al., 2013; Linck et al., 2010). There are also observations suggesting that this oil activates positive affect in rodents and humans (Frasnelli et al., 2015). To our knowledge, there are no data concerning possible modifications of the effects of lavender oil by ovarian hormones.

An additional purpose of the present study was to evaluate the effects of the positive stimuli lavender oil, music, and chocolate on estradiol + progesterone-activated sexual behavior in ovariectomized rats and to determine if and how non-sexual responses were modified by ER ligands.

We have previously argued that an understanding of the behavioral consequences of the central nervous actions of the ovarian hormones is best achieved in experimental setups with external validity (Chu and Ågmo, 2014; Chu and Ågmo, 2015b; Chu and Ågmo, 2016). This means that the setup should include as many as possible of the elements found in the natural context in which the behavior normally is shown. In the case of sexual behaviors in rats, an important feature is that it occurs in multi-male, multi-female groups, and in a physical environment making it possible for the rats to temporarily escape from other group members.

In view of these considerations, we administered estradiol as well as the selective ERα agonist propylpyrazoletriol (PPT) and the selective ERβ agonist diarylpropionitrile (DPN) to ovariectomized female rats housed in a seminatural environment in groups consisting of 4 females and 3 males. During the period in which the agonists could be expected to have their maximal effect, we introduced the events mentioned earlier into the environment. This made it possible not only to determine the role of the ERs in social and sexual interactions in a group of rats, but also how they affected the response to these events, and how the positive and negative events themselves affected behavior. These data would provide us with a better understanding of how the ERs control sexual behavior and responses to emotion-inducing events in a procedure with external validity.

2. Material and methods

2.1. Subjects

Wistar rats (females, 250 g and males 300 g upon arrival) were obtained from Charles River WIGA (Sulzfeld, Germany). The rats were housed in same-sex pairs in standard Macrolon® IV cages prior to the beginning of the experiment. Commercial rat pellets (RM1, Special Diets Services, Witham, UK) and tap water were available ad libitum. The animal rooms were maintained at 21 ± 1 °C and humidity was 55 ± 10%. Lights were set on a reversed 12:12 h cycle, being on between 23:00 and 11:00 h. Females were ovariectomized 14 days prior to the introduction into the seminatural environment under isofluorane anesthesia. For a detailed description of the surgical procedure see Ågmo (1997).

All experimental procedures employed in the present experiment were approved by the Norwegian Food Safety Authority and were in agreement with the European Union council directive 2010/63/EU.

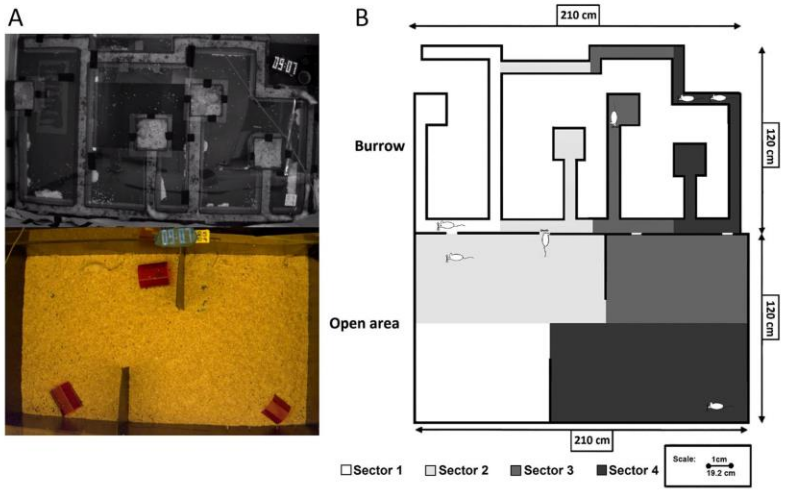

Fig. 1. A. Picture of the seminatural environment. B. The division in zones.

2.2. Apparatus

The seminatural environment has previously been used in a number of studies and has been described in detail elsewhere (Chu and Ågmo, 2014; Chu and Ågmo, 2015b). Briefly, it consisted of a complex burrow system and a large open area (Fig. 1). The burrow system included four nest boxes provided with nest material and was maintained in complete darkness for the whole experiment. Infrared (850 nm) lamps provided the light necessary for video recording. The open area (1.2 ∗ 2 m) contained 12 wooden sticks and three small shelters made of transparent red plastic. The open field was submitted to a reversed light/dark cycle (12D:12L) with a 180 lx light from 23:00 to 11:00 and a 30 lx light from 11:00 to 23:00. Artificial dusks and dawns were provided by slowly changing light intensity from night to day and day to night during a 30 min period preceding and following the light period. Video cameras were fixed to the ceiling about 2 m above the burrow and the open area, respectively. They were connected to digital video recorders.

The ventilation system in the animal facility produced an ambient noise of about 40 dB.

2.3. Hormones and selective estrogen receptor ligands

Estradiol benzoate (EB) and progesterone (P) (both from Sigma Aldrich, St Louis, MO) were dissolved in peanut oil (Den norske Eterfabrikk, Norway) and administered subcutaneously (SC) 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 progesterone. EB was administered 48 h before P.

The estrogen receptor agonists propylpyrazoletriol (PPT) and diarylpropionitrile (DPN) were obtained from Tocris Bioscience, Bristol, UK. Both PPT and DPN were dissolved in undiluted dimethylsulfoxide (Sigma Aldrich) right before SC injection. Both were administered at a dose of 2 ∗ 10 mg/kg body weight in a volume of 1 ml/kg. There was an interval of 24 h between injections. PPT is selective to ERα with a 410-fold preference compared to ERβ, and with a relative binding affinity of 50% compared to estradiol (Stauffer et al., 2000). DPN is selective to the ERβ with a 72-fold preference compared to ERα with a relative binding affinity of 18% (Meyers et al., 2001). PPT and DPN reach their maximum serum concentration about 30 min after SC injection and have a half-life of 6.0 ± 0.03 h and 8.2 ± 1.7 h, respectively (Sepehr et al., 2012).

The doses of EB and P employed here have been used successfully in several earlier studies (e.g. Chu et al., 2017; Snoeren et al., 2015). They produce close to maximal receptivity and high intensity of paracopulatory behaviors (Spiteri and Ågmo, 2006). The dose of PPT was based on earlier studies. One showed that 2 ∗ 10 mg/kg of PPT given 48 and 24 h before test produced a high lordosis quotient, similar to that of 2 ∗ 2 μg/rat of EB. A dose of 2 mg/kg was inactive (Miller et al., 2005). Another study showed that 2 ∗ 5 mg/rat, 48 h and 24 h before test, produced a lordosis quotient of about 0.8 when combined with progesterone 0.5 mg/rat (Mazzucco et al., 2008). Thus, a dose of 2 ∗ 10 mg/kg of PPT was used in order to assure clear behavioral effects. Concerning DPN, the dose was chosen somewhat arbitrarily. The ERβ does not participate in the activation of sexual behavior, so we needed to find another basis for determining the appropriate dose. Since many studies comparing the effects of PPT and DPN employ the same dose of both compounds (e.g. Pisani et al., 2016; Walf and Frye, 2005) we simply decided to do the same.

2.4. Experimental conditions

Since the aims of this study include an analysis of behavior in situations producing positive and negative affect, it was essential to introduce events into the seminatural environment that reasonably could be expected to produce diverse emotional reactions. Five such events, all mentioned in the Introduction, were used.

1. Lavender odor stemmed from an essential oil extracted from *Lavandula angustifolia* (AromaBio, Lyon, France). Immediately before the beginning of the experimental session, 1.5 ml of this oil was put on a cotton pad in an airtight jar. An air stream could be made to flow through the jar whenever desired with the help of an air pump and a system of solenoid valves (Olfactory Stimulus Package, Medical associates, Georgia, Vt). Odorless plastic tubing (I.D. 3/16″, O.D. 5/16″, wall thickness 1/16″; TYGON® Inert, Saint-Gobain Verneret, Charny, France) connected the pump with the valves and eventually with a nozzle in the wall of one of the tunnels in the burrow section of the seminatural environment, as well as with another nozzle in the wall of the open area. The airflow (around 3 l/min) could be directed through one of two jars, or made to bypass the jars and consist of unscented room air instead. Room air was blown through the nozzles during the entire experimental session, except when it was replaced by an olfactory stimulus. Lavender odor was introduced into the seminatural

environment for 30 min with the help of this system. The amount of lavender oil used here has been found to alter behavior even with short exposure times (e.g. Shaw et al., 2007).

2. Classical music (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). The piece lasts 24 min and 18 s. The music file was uploaded on a computer and played through A60 stereo speakers from Creative (Clas Ohlson, Norway) at a sound intensity of 55–60 dB as measured at floor level.

3. Thirty-five one g chocolate pellets (Bioserve, *Flemington, NJ*) were placed on a Petri dish (diameter 100 mm) which was put in the middle of the open area. After 30 min, the dish was removed. At that time, most pellets had been removed by the rats.

4. A 90 dB white noise was produced by a noise generator (Lafayette instruments, Lafayette, IN) connected to two loudspeakers (Scan-Speak Discovery 10F/8414G10, Hifi Kit Electronic, Stockholm), one suspended about 2 m above the burrow, and another at the same height above the open area. The noise was turned on for a period of 15 min.

5. Fox odor was produced by making air flow through a jar containing 35 µl of 2,5-dihydro-2,4,5-trimethylthiazoline (TMT; Contech, Delta, BC, Canada) on a cotton pad. The odor system described above was used. Short exposure to odor from this amount of TMT has been found to alter behavior in a large open field (Morrow et al., 2002). The odor exposure lasted for 30 min.

Music and chocolate may not be standard parts of rats' natural habitat, and these stimuli might therefore reduce external validity. We used them because they are among the few, non-sexual stimuli, known to cause positive affect in non-deprived rats. Sucrose might have been used instead of chocolate pellets, but the latter have the advantage of allowing for quantification of each individual's consumption. This would not have been possible with a sucrose solution.

2.5. Procedure

Prior to introducing each experimental group into the seminatural environment, the floor of the entire environment was covered with a 3 cm thick layer of aspen wood chips (Tapvei, Harjumaa, Estonia). Four 0.5 l water bottles and about 5 kg of standard food pellets were located in a corner of the open area. After each experimental session, the bedding was removed and the entire environment was cleaned and disinfected.

The rats were released in the seminatural environment on day 0 at 13:00. About 4 h before, they had been weighed and marked in order to be identifiable on the video record.

The rats were left undisturbed for the first 5 days in the seminatural environment. On days five and six the females were captured and injected with the appropriate compound. On day 7, all females received P. Four hours later, the sequence of experimental conditions started. All experimental conditions were separated by a 50-minutes rest period (Table 1). The experiment was terminated after the last experimental condition, and the females were again weighed.

The order of presentation of the experimental conditions was fixed throughout the whole experiment. Four hours after the P injection, lavender odor was presented, followed by music, chocolate, white noise and the fox odor.

This order of events was based on several considerations. Predator odor has been reported to alter behavior for several hours (Fendt et al., 2005). Consequently, exposure to fox odor needed to be the last condition. The duration of potential effects of lavender oil is not known, but anxiolytic effects are normally obtained immediately or within few minutes after the end of exposure (e.g. Tsang et al., 2013). The ventilation system in the room housing the seminatural environment assures 15 air changes per hour, leading to a fast decline in the concentration of odorant. We found it reasonable to suppose that the 50 min interval before the next condition would be sufficient both for the odor and for its possible effects to dissipate. In fact, the data confirmed that supposition. Concerning music, the reported effects were usually obtained during exposure (e.g. Escribano et al., 2014). There are no data as to the duration of effect, but again we supposed that it should be < 50 min after the end of the piece. The duration of the positive affect produced by chocolate eating or of the fear reaction produced by white noise has not been determined. It may be pointed out, though, that the chocolate-induced positive affect is present already during consumption (La Mela et al., 2010; Reynaert et al., 2016). With regard to white noise, pilot data revealed that the behavioral effects of the noise were most evident at the onset, and that behavior began to normalize already during the last few minutes of noise exposure. Therefore, the 50 min interval was probably sufficient for any noise effects to dissipate.

2.6. Design

Each group in the seminatural environment consisted of seven rats, four females and three males. The group members came from different cages, meaning that they were unknown to each other before the introduction into the environment. Ten such groups were used in this experiment.

In all groups, each of the four females received a different treatment. 1. Oil on days 5 and 6, P on day 7. 2. EB on day 5, oil on day 6, and P on day 7. 3. PPT on days 5 and 6, P on day 7. 4. DPN on days 5 and 6, P on day 7. This means that all treatments were present in all housing groups.

2.7. Behavioral observations

Based on extensive observation of the video record, we established an ethogram for the scoring of the rats' behavior (Table 2). Scoring was made with the Observer XT 12.5 software (Noldus, Wageningen, the Netherlands). Pilot data showed that a 15 min observation period was sufficient to detect behavioral differences between conditions and treatments. Thus, the last 15 min of the lavender, music, and fox odor exposure were observed, whereas we recorded behaviors for the first 15 min of chocolate availability. This allowed us to determine the latency to approach and grab the chocolate. The entire 15 min period of exposure to white noise was observed. Behavior during the 15 min preceding the lavender odor was recorded as a baseline. The frequency and/or duration of the occurrences of the behavior patterns were

Table 1
Summary of the experimental procedure on the test day.

Experimental condition					
Baseline	Lavender odor	Music	Chocolate	White noise	Fox odor
Time of the day					
12:30–13:00	13:00–13:30	14:20–14:44	15:35–16:05	16:55–17:10	18:00–18:30
Observation time					
12:45–13:00	13:15–13:30	14:29–14:44	15:35–15:50	16:54–17:09	18:15–18:30
Duration					
30 min	30 min	24 min 18 s	30 min	15 min	30 min

Table 2
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.
Female attractivity	*Rejection; f*	Female kicks, boxes or assumes a belly up posture.
	Mounts received; f	Male catches the female by her waist and puts his belly over her back, with pelvic thrusting.
	Male pursuit; f,d	Male runs after a female with his snout in close to the anogenital zone of the female.
Prosocial behaviors	*Male sniffing female; f,d*	Snout close to a female, sniffing the fur.
	Male sniffing anogenital area, f,d	Male sniffs the anogenital zone of a female by putting his snout under her tail.
	Resting with other females; f,d	Rests immobilized in relaxed position at a distance shorter than one rat to one or several females.
	Resting with males, f,d	Rests immobilized in relaxed position at a distance shorter than one rat to 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.
Antisocial behaviors	*Hiding with another rat*[b]*; f,d*	Immobilized in a corner or in a nest box within one body length of the other rat.
	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.
Solitary behaviors	*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.
	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*	Chew on chocolate.
	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.
Exploratory behaviors and ambulatory activity	*Flee from noise*[b]*; o,l*	Rush 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.
	Sniffing the floor, f,d	Sniffs the floor material with all four paws on the floor.
	Rearing; f,d	Sniffs the air while standing on the hind legs.
	Transitions; f	Displays a behavior in a zone different from the one in which the previous behavior was displayed.

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

recorded, specifying the individual initiating the behavior, the individual to whom it was directed, and the location of the behavior. We also calculated the number of transitions between zones (see Fig. 1), as well as the number of visits to and the time spent in the open area. In the case of latencies, subjects not displaying the behavior were assigned a latency of 900 s, the duration of the observation. The lordosis quotient (LQ, number of lordoses/number of mounts) was also calculated. Please note that some females displayed one or more lordoses in the absence of male mounting, usually in response to tactile stimulation of posterior body parts. The LQ may, consequently, be larger than 1.

2.8. Statistical analysis

Whenever possible, data were analyzed with two-factor ANOVA for repeated measures on one factor. The between-groups factor was treatment and the within-groups factor was experimental condition. After significant main effect, the Tukey HSD test was used for a posteriori comparisons. We calculated the effect size η^2 for the effect of treatment and the partial effect size $\eta_p{}^2$ for effect of experimental condition and for the interaction. The effect size for Tukey's HSD was expressed as Cohen's d ($d = (x_1 - x_2) / \sigma$).

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 F_{max} test, we used nonparametric tests. The effect of treatment was evaluated with the Kruskal-Wallis test whereas the effect of experimental condition was analyzed with Friedman's ANOVA. In case of significance, post hoc analyses were made as recommended by Conover (Conover, 1999). The effect size was calculated as eta squared ($\eta_H{}^2$) for the Kruskal-Wallis tests and as Kendall's *W* for the Friedman test (Tomczak and Tomczak, 2014). Cliff's δ was used for the non-parametric post hoc comparisons (Cliff, 1996). Some data were analyzed with the χ^2 test, and/or the Fisher exact test. Effects sizes for these tests were calculated with Cramer's V and Cohen's d, respectively.

Significance level was $p < 0.05$. Data in text and figures are expressed as mean + SEM. The IBM SPSS Statistics, version 23 was used for parametric tests and the free software R, version 3.4.3 with base, PMCMRplus, effsize and lsr packages for non-parametric tests.

2.9. Co-occurrence analysis

The seminatural environment allows the subjects to express a substantial part of their natural behavioral repertoire. In fact, the continuous flow of behavior patterns is recorded. This makes it possible to determine treatment- or condition-induced modifications of that flow. In other words, how the experimental manipulations might have altered the structure of behavior. Analyses of the frequency or duration of particular behavioral items cannot reveal this kind of effects. Thus, in order to fully exploit the data obtained, we subjected the behavioral record to an analysis of co-occurrence. Since the behavior patterns were recorded in chronological order, this is easily made. We used a moving window of four behavior patterns, and determined how often one behavior pattern occurred together with another in the same window. This is defined as a co-occurrence. The window moved, by steps of one behavior pattern, over the entire individual record. The frequency of co-occurrences was entered in a matrix with the behavior patterns in rows and columns, the co-occurrence frequency appearing at the intersections. Treatments and experimental conditions were also included in the matrix. These were the raw data for the analysis. Descending hierarchical classification was used in order to find clusters of related behavior (Reinert, 1983; Reinert, 1990; Valax et al., 1990, see also

LePape et al., 1997). The descending hierarchical classification is based on the probability for an item to be proportionally more present in a cluster than it is in the entire data set, as evaluated by χ^2 analysis. Each item is permutated from one cluster to the other to test the robustness of the classification, until statistically independent profiles of items appear (Marchand and Ratinaud, 2012). Communities can therefore be interpreted as groups of individuals and behaviors significantly more co-occurring together than with items of another community.

The criterion for including elements in their respective classes is a higher frequency compared to the average occurrence, as well as an association with the class determined by χ^2 values equal to or higher than 3.84. This gives an error margin of 0.05 when df = 1 (de Oliveira Andrade and de Oliveira Andrade, 2016).

Finally, co-occurrence networks were established and visualized using the Fruchterman-Reingold algorithm. Calculations were performed with the free software IRAMUTEQ (Interface de R pour les Analyses Multidimensionnelles de Textes et de Questionnaires; available at http://www.iramuteq.org/).

3. Results

The pattern of effects of experimental condition and of treatment were similar for the frequency of recorded behaviors and the total duration as well as the mean duration of each behavioral episode, whenever these could be calculated. Therefore, we only present frequency data. These have the advantage of being available for all behaviors.

3.1. Effects of experimental conditions

3.1.1. Female sexual behaviors

The sex behavior data did not satisfy the criteria for ANOVA. The distribution greatly deviated from normality according to the Shapiro-Wilk test and Hartley's F_{max} test showed error variances to be non-homogenous. Therefore, these data were analyzed using non-parametric statistics. Moreover, since the aim of these analyses was to determine how experimental conditions affected sexual behaviors, we limited the analyses to females treated with EB or PPT. The females treated with oil or DPN expressed an extremely low level, or none at all, of these behaviors (see 3.2.1). Thus, these females could not contribute with any useful data to an analysis of the effects of experimental condition on sexual behaviors, since no such behaviors were displayed in any condition.

The lordosis frequency in the collapsed EB and PPT groups differed between conditions ($\chi^2_{DF=5} = 12.67$, $p = 0.027$, W = 0.12). It was lower during exposure to white noise ($p = 0.023$, $\delta = 0.30$) than at baseline (Fig. 2A). The LQ also differed between experimental conditions ($\chi^2_{DF=5} = 15.56$, $p = 0.008$, W = 0.16), being lower during exposure to white noise ($p = 0.002$, $\delta = 0.45$) than at baseline (Fig. 2B). Likewise, the frequency of paracopulatory behaviors differed between conditions ($\chi^2_{DF=5} = 15.57$, $p = 0.008$, W = 0.16). It was lower during exposure to white noise ($p = 0.004$, $\delta = 0.37$) than at baseline (Fig. 2C). The frequency of rejection did not vary between the experimental conditions ($\chi^2_{DF=5} = 1.72$, $p = 0.887$, W = 0.02; Fig. 2D).

3.1.2. Female attractivity to males

Behaviors indicative of female attractiveness were analyzed using non-parametric statistics due to lack of normality. Here, all treatments were included since also the females treated with Oil or DPN were somewhat attractive to the males. The number of mounts received by the females was affected by the experimental conditions ($\chi^2_{DF=5} = 12.20$, $p = 0.032$, $W = 0.32$), but none of the conditions differed from baseline ($ps > 0.092$; data not shown). The frequency of male pursuit of the females also differed between the experimental conditions ($\chi^2_{DF=5} = 19.07$, $p = 0.002$, $W = 0.50$). The pursuit frequency was lower during exposure to fox odor than at baseline ($p = 0.043$, $\delta = 0.17$). The other conditions had no effect on the frequency of pursuit (all $ps > 0.270$; Fig. 3A).

The frequency of male anogenital sniffing of the females did not vary between the experimental conditions ($\chi^2_{DF=5} = 8.27$, $p = 0.142$, $W = 0.05$) and there was no meaningful effect on male resting with females (data not shown). To the contrary, the frequency of male sniffing of the females differed between the experimental conditions ($\chi^2_{DF=5} = 16.85$, $p = 0.005$, $W = 0.09$). Males sniffed females more often during exposure to chocolate than at baseline ($p = 0.019$, $\delta = 0.34$; Fig. 3B).

3.1.3. Exploratory behavior

Ambulatory activity, expressed as the frequency of transition between zones in the entire seminatural environment, differed between the experimental conditions ($F_{5170} = 10.59$, $p < 0.001$, $\eta_p^2 = 0.24$). Lavender ($p < 0.05$, d = 0.62), chocolate ($p < 0.05$, d = 0.92) and white noise ($p < 0.001$, d = 0.80) enhanced activity, whereas exposure to music and TMT had no effect ($ps > 0.06$; Fig. 4A).

The transitions between zones in the open area also differed between experimental conditions ($F_{5170} = 10.17$, $p < 0.001$, $\eta_p^2 = 0.23$). Exposure to lavender odor ($p < 0.05$, d = 0.67) and to chocolate ($p < 0.05$, d = 0.63) increased activity. The other conditions had no effect ($ps > 0.166$; Fig. 4B). The number of transitions between zones in the burrow was also affected by experimental condition ($F_{5170} = 17.34$, $p < 0.001$, $\eta_p^2 = 0.34$). Exposure to chocolate ($p < 0.05$, d = 1.04) and white noise ($p < 0.05$, d = 1.13) increased ambulatory activity in the burrow compared to baseline (Fig. 4C).

There was an effect of experimental condition on the time spent in the open area ($F_{5170} = 7.73$, $p < 0.001$, $\eta_p^2 = 0.19$). Music ($p < 0.05$, d = 0.65) and white noise ($p < 0.05$, d = 0.62; Fig. 4D) reduced the time spent in the open area compared to baseline, whereas the other conditions had no effect. Logically, the time spent in the burrow was also affected by experimental condition ($F_{5170} = 7.73$, $p < 0.001$, $\eta_p^2 = 0.19$) in a way opposite to the open area. Music ($p > 0.05$, d = 0.65) and white noise ($p < 0.05$, d = 0.62) enhanced the time spent in the burrow (Fig. 4E).

The frequency of rearing was also modified by the experimental condition ($F_{5170} = 5.81$, $p < 0.001$, $\eta_p^2 = 0.15$). More rearing episodes were observed during exposure to white noise than at baseline ($p < 0.05$, d = 0.70; Fig. 4F). The experimental conditions also altered the frequency of sniffing the floor ($F_{5170} = 26.57$, $p < 0.001$, $\eta_p^2 = 0.44$). Exposure to chocolate ($p < 0.05$, d = 1.00) and white noise ($p < 0.05$, d = 1.42) enhanced the frequency relative to baseline (Fig. 4G).

3.1.4. Female prosocial behaviors

These behaviors could, obviously, be directed towards the other females in the group or to the males. We found it useful to analyze female-female and female-male interactions separately. The frequency of resting with another female did not change between experimental conditions ($F_{(5,170)} = 2.19$, $p = 0.058$, $\eta_p^2 = 0.06$). To the contrary, the time resting with males differed between experimental conditions ($F_{(5,170)} = 23.88$, $p < 0.001$, $\eta_p^2 = 0.41$) The females rested more with males during exposure to chocolate than at baseline ($p < 0.05$, d = 0.70). These data are illustrated in Fig. 5A. We also found main effects of experimental condition both on the frequency of female sniffing another female ($F_{5170} = 12.66$, $p < 0.001$, $\eta_p^2 = 0.27$) and a male ($F_{5170} = 10.37$, $p < 0.001$, $\eta_p^2 = 0.23$). The frequency was always higher during exposure to chocolate than at baseline (female-female, $p < 0.05$, d = 0.56; female-male, $p < 0.05$, d = 0.53). Data are shown in Fig. 5B.

3.1.5. Female antisocial behavior towards males and females

Only the female nose-off behavior satisfied the criteria for parametric analysis. All the other antisocial behaviors were analyzed using non-parametric statistics.

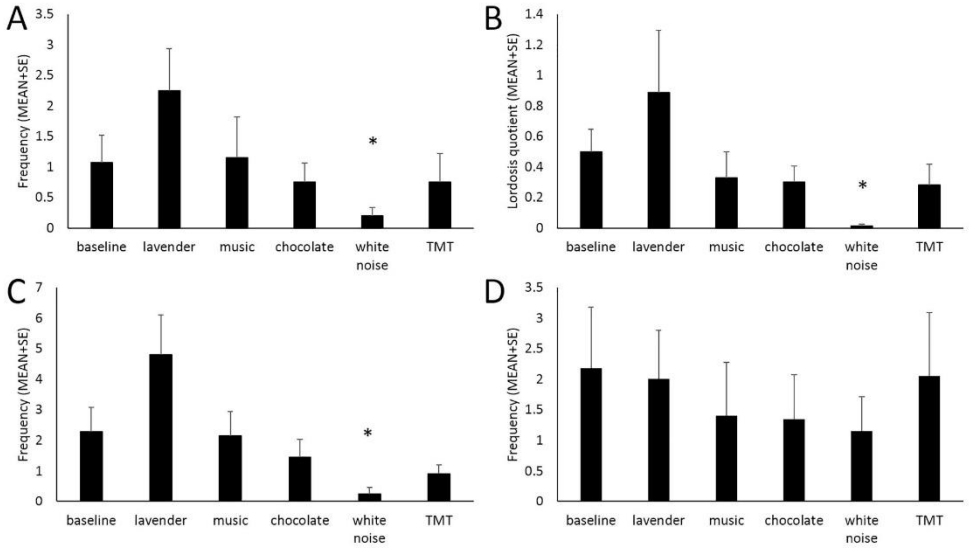

Fig. 2. Effect of the experimental conditions on female sexual behavior, for EB-and PPT-treated groups only, both treatments collapsed. A. Lordosis frequency. B. Lordosis quotient. C. Frequency of paracopulatory behaviors. D. Rejection frequency. Data are mean ± SEM. Friedman test, post hoc: Conover-Iman test. *, different from baseline. N = 20.

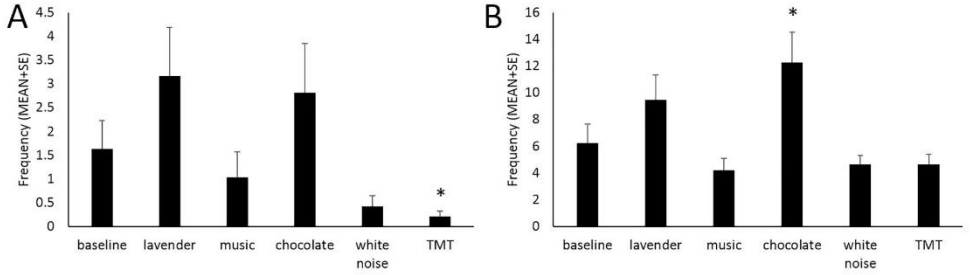

Fig. 3. Effect of the experimental conditions on female attractivity to males, all treatments collapsed. A. Pursuit frequency. B. Frequency of male sniffing of a female. Data are mean ± SEM. Friedman test, post hoc: Conover-Iman test. *, different from baseline. N = 38.

The nose-off frequency varied between the experimental conditions regardless of the sex of the other party (males, $F_{5170} = 3.95$, $p = 0.002$, $\eta_p^2 = 0.10$; females, $F_{5170} = 7.32$, $p < 0.001$, $\eta_p^2 = 0.18$). More nose-off episodes occurred during exposure to chocolate than at baseline in both cases (males, $p < 0.05$, d = 0.35; females, $p < 0.05$, d = 0.61). White noise increased nose-off of other females ($p < 0.05$, d = 0.97) but not of males ($p > 0.05$, d = 0.24). Data are found in Fig. 6A. As can be seen in Fig. 6B, the frequency of fleeing from the males as well as from other females differed between conditions (males, $\chi^2_{DF=5} = 29.89$, $p < 0.001$, W = 0.16; females, $\chi^2_{DF=5} = 48.90$, $p < 0.001$, W = 0.26). The fleeing frequency was higher during exposure to chocolate and white noise than at baseline (males, chocolate-baseline: $p = 0.001$, $\delta = 0.48$; white noise-baseline: $p < 0.001$, $\delta = 0.41$; females, chocolate-baseline: $p = 0.001$, $\delta = 0.52$; white noise-baseline: $p < 0.001$, $\delta = 0.58$).

3.1.6. Non-social behaviors

There was no systematic effect of experimental condition on drinking, self-grooming or resting alone (data not shown).

3.1.7. Summary of the effects of experimental condition

The only experimental condition with an effect on the display of female sexual behaviors was white noise. The stimulus reduced these behaviors. The other conditions were ineffective with regard to sex behaviors, and none of the conditions modified female attractivity. The availability of chocolate pellets stimulated ambulatory activity throughout the seminatural environment and enhanced both prosocial and antisocial interactions with both sexes. White noise was associated with avoidance of the open area and increased ambulatory activity in the burrow only. Antisocial behaviors were also enhanced in this condition. The other purportedly fear-inducing stimulus, fox odor, had no consistent effect. This was also the case for lavender odor and music.

3.2. Effects of hormone treatment

3.2.1. Female sexual behaviors

When the treatment effect was collapsed over all experimental conditions, it was found that the frequency of lordosis differed between treatments ($H_{3,\ N=38} = 22.71$, $p < 0.001$, $\eta_H^2 = 0.60$). The Oil group displayed less lordoses than the EB group ($p < 0.001$, $\delta = 0.90$) and the PPT group ($p = 0.001$, $\delta = 0.60$). There was no difference between

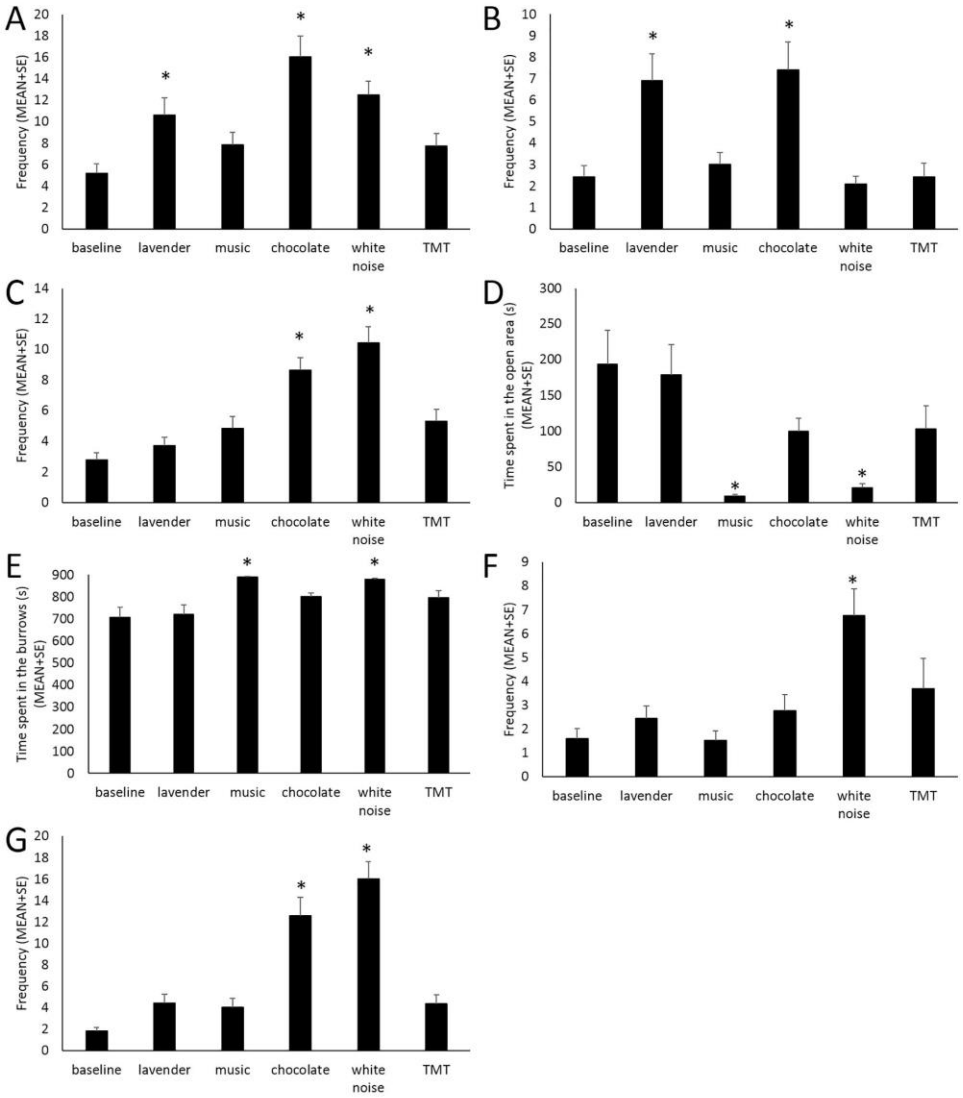

Fig. 4. Effect of the experimental conditions on female exploratory behavior, all treatments collapsed. A. Frequency of transition between the zones of the seminatural environment. B. Frequency of transition between the zones of the open area. C. Frequency of transition between the zones of the burrow system. D. Time spent in the open area. E. Time spent in the burrow system. F. Rearing frequency. G. Frequency of sniffing the floor. Data are mean ± SEM. Repeated measures two-way ANOVA, post hoc: Tukey HSD. *, different from baseline. N = 38.

the Oil group and the DPN group ($p = 1$, $\delta = 0.00$). When evaluating treatment effects within each of the experimental conditions it was found that the number of lordoses differed between treatments at baseline ($H_{3,\ N=38} = 10.34$, $p = 0.016$, $\eta_H^2 = 0.27$), during exposure to lavender odor ($H_{3,\ N=38} = 10.42$, $p = 0.015$, $\eta_H^2 = 0.27$), music ($H_{3,\ N=38} = 12.14$, $p = 0.007$, $\eta_H^2 = 0.32$) and chocolate ($H_{3,\ N=38} = 19.25$, $p < 0.001$, $\eta_H^2 = 0.51$). No difference between the treatments was observed during exposure to the negative conditions, white noise ($H_{3,\ N=38} = 5.76$, $p = 0.124$, $\eta_H^2 = 0.15$) and TMT odor ($H_{3,\ N=38} = 6.30$, $p = 0.098$, $\eta_H^2 = 0.17$). The EB group showed more lordoses than the Oil group at baseline ($p = 0.006$, $\delta = 0.50$), during exposure to lavender odor ($p = 0.038$, $\delta = 0.40$), music ($p = 0.003$, $\delta = 0.40$) and chocolate ($p < 0.001$, $\delta = 0.60$). White noise and TMT odor suppressed responding, since the EB and Oil groups did no longer differ. The PPT group displayed more lordoses than the Oil group only during exposure to lavender odor ($p = 0.009$, $\delta = 0.500$). DPN failed to stimulate lordosis in all conditions. Results are illustrated in Fig. 7A.

There was a treatment effect on the LQ ($H_{3,\ N=38} = 13.50$, $p = 0.004$, $\eta_H^2 = 0.36$) when all experimental conditions were collapsed. Only the EB group differed significantly from Oil ($p = 0.001$,

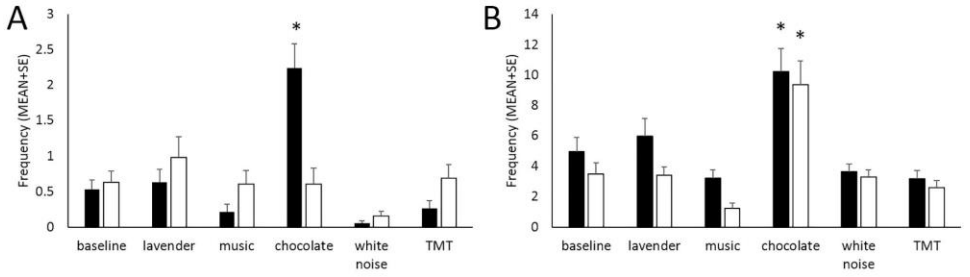

Fig. 5. Effect of the experimental conditions on female social behavior directed to males (black) and to other females (white), all treatments collapsed. A. Frequency of resting with another rat. B. Frequency of sniffing another rat. Data are mean ± SEM. Repeated measures two-way ANOVA, post hoc: Tukey HSD. *, different from baseline. N = 38.

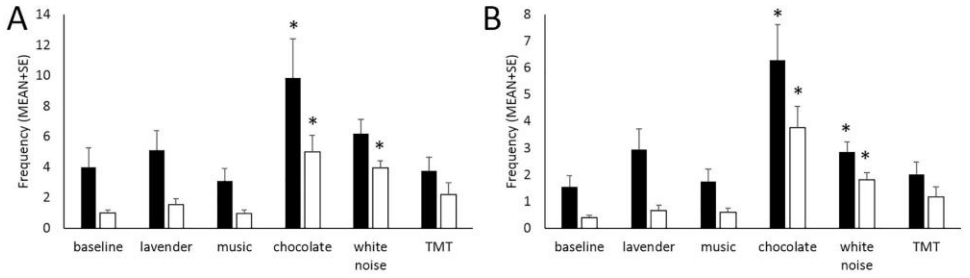

Fig. 6. Effect of the experimental conditions on female anti-social behavior directed to males (black) and to other females (white), all treatments collapsed. A. Nose-off frequency; repeated measures two-way ANOVA, post hoc: Tukey HSD. B. Frequency of fleeing from another rat; Friedman test, post hoc: Conover-Iman test. Data are mean ± SEM. *, different from baseline; N = 38.

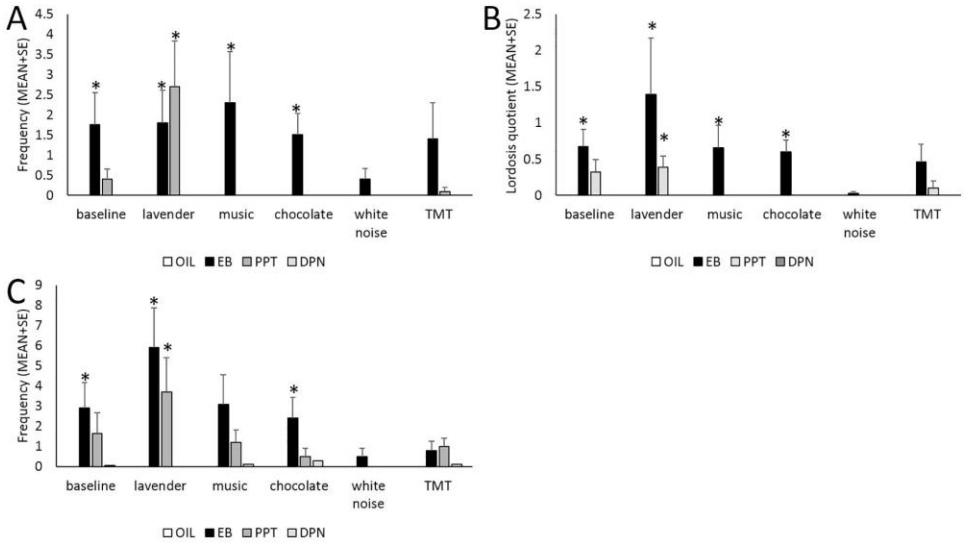

Fig. 7. Effect of the treatment on female sexual behavior. A. Lordosis frequency. B. Lordosis quotient. C. Frequency of paracopulatory behaviors. Kruskal-Wallis test, post hoc: Conover test. Data are mean ± SEM. *, different from the Oil group. Oil: n = 8, EB: n = 10, PPT: n = 10, DPN: n = 10.

δ = 0.60). Treatment with PPT failed to significantly enhance the LQ ($p = 0.053$, δ = 0.40), whereas DPN was completely inactive ($p = 1$, δ = 0). The treatment effect was absent during exposure to white noise ($H_{3,\ N=38} = 2.80$, $p = 0.424$, $\eta_H^2 = 0.07$) or fox odor ($H_{3,\ N=38} = 6.30$, $p = 0.098$, $\eta_H^2 = 0.17$), but present in all other conditions (baseline, $H_{3,\ N=38} = 10.36$, $p = 0.016$, $\eta_H^2 = 0.27$; lavender odor, $H_{3,\ N=38} = 10.81$, $p = 0.013$, $\eta_H^2 = 0.29$; music, $H_{3,\ N=38} = 12.14$, $p = 0.007$, $\eta_H^2 = 0.32$; chocolate, $H_{3,\ N=38} = 19.43$, $p < 0.001$,

$\eta_H^2 = 0.51$). During these conditions, EB-treated females systematically had a higher LQ than oil-treated females (baseline: $p = 0.006$, $\delta = 0.50$; lavender: $p = 0.006$, $\delta = 0.50$; music: $p = 0.003$, $\delta = 0.40$; chocolate: $p < 0.001$, $\delta = 0.60$). Exclusively during exposure to lavender, the PPT group displayed a higher LQ than the Oil group ($p = 0.050$, $\delta = 0.40$). Data are illustrated in Fig. 7B.

There was also an effect of treatment on the frequency of paracopulatory behaviors when all experimental conditions were included in the analysis ($H_{3,\ N=38} = 21.06$, $p < 0.001$, $\eta_H^2 = 0.55$). The Oil group displayed less paracopulatory behaviors than the EB group ($p < 0.001$, $\delta = 0.90$) and the PPT group ($p < 0.001$, $\delta = 0.80$). There was no difference between the Oil group and the DPN group ($p = 0.273$, $\delta = 0.30$).

We then proceeded to evaluate treatment effects on paracopulatory behavior under each of the experimental conditions. There was a difference between treatments at baseline ($H_{3,\ N=38} = 10.34$, $p = 0.016$, $\eta_H^2 = 0.27$), during exposure to lavender odor ($H_{3,\ N=38} = 12.68$, $p = 0.005$, $\eta_H^2 = 0.33$) and chocolate ($H_{3,\ N=38} = 11.84$, $p = 0.021$, $\eta_H^2 = 0.26$). No difference was found between the treatments in the frequency of paracopulatory behaviors during the exposure to music ($H_{3,\ N=38} = 6.95$, $p = 0.074$, $\eta_H^2 = 0.18$), white noise ($H_{3,\ N=38} = 5.75$, $p = 0.124$, $\eta_H^2 = 0.15$) and fox odor ($H_{3,\ N=38} = 7.46$, $p = 0.059$, $\eta_H^2 = 0.20$). The EB group showed more paracopulatory behaviors than the Oil group at baseline ($p = 0.003$, $\delta = 0.60$), during exposure to lavender odor ($p = 0.002$, $\delta = 0.60$) and chocolate ($p = 0.003$, $\delta = 0.60$). The PPT group displayed more paracopulatory behaviors than the Oil group during exposure to lavender odor ($p = 0.038$, $\delta = 0.40$) but not during the other conditions. The DPN group did not differ from the Oil group in any condition. These data are found in Fig. 7C. The frequency of rejections was not modified by the treatments ($H_{3,\ N=38} = 3.30$, $p = 0.347$, $\eta_H^2 = 0.09$; data not shown).

3.2.2. Female attractivity to males

All experimental conditions collapsed, there was a main effect of treatment on the number of mounts received ($H_{3,\ N=38} = 20.03$, $p < 0.001$, $\eta^2 = 0.53$). The females in the EB group were mounted more than the females in the Oil group ($p < 0.001$, $\delta = 0.90$) and so were females treated with PPT ($p = 0.024$, $\delta = 0.40$). The DPN group ($p = 0.678$, $\delta = 0.10$) did not differ from the Oil group.

The number of mounts received differed between treatments during exposure to lavender odor ($H_{3,\ N=38} = 10.25$, $p = 0.017$, $\eta_H^2 = 0.27$), music ($H_{3,\ N=38} = 8.86$, $p = 0.031$, $\eta_H^2 = 0.23$) and chocolate ($H_{3,\ N=38} = 15.60$, $p = 0.001$, $\eta_H^2 = 0.41$). No difference was found at baseline, during exposure to white noise or fox odor (all $ps > 0.103$). The EB group received more mounts than the Oil group during exposure to lavender odor ($p = 0.012$; $\delta = 0.50$), music ($p = 0.016$; $\delta = 0.30$) and chocolate ($p < 0.001$, $\delta = 0.50$). Only during exposure to lavender odor, females treated with PPT were more mounted than those treated with Oil ($p = 0.031$; $\delta = 0.50$). The DPN group was never different from the Oil group (all $ps = 1$). Data are summarized in Fig. 8A.

All experimental conditions collapsed, there was an effect of the treatment on the frequency of male pursuit of the females ($H_{3,\ N=38} = 13.73$, $p = 0.003$, $\eta_H^2 = 0.36$). The males pursued females given EB more than those given oil ($p = 0.002$, $\delta = 0.78$). Neither PPT- nor DPN-treated females differed from Oil ($ps > 0.120$).

The number of pursuit episodes differed between the treatments during exposure to lavender odor ($H_{3,\ N=38} = 8.24$, $p = 0.041$, $\eta_H^2 = 0.22$). There was no difference between the treatments in the frequency of male pursuit at baseline, during exposure to music, chocolate, white noise and fox odor (all p's > 0.053). During exposure to lavender odor the EB group was more pursued by the males than the Oil group ($p = 0.017$, $\delta = 0.55$). The other treatment groups were not different from the Oil group (all $ps > 0.129$; Fig. 8B).

The frequency of male anogenital sniffing of the females was unaffected by the treatments ($H_{3,\ N=38} = 4.67$, $p = 0.198$, $\eta_H^2 = 0.12$) when all experimental conditions were considered. We then proceeded with an analysis of the effects of EB in each of the experimental conditions. It turned out that the frequency of male anogenital sniffing did not differ between treatments at any of the experimental conditions ($ps > 0.058$). Neither the frequency of male resting with females nor the frequency of sniffing the females differed between treatments at any of the experimental conditions (all $ps > 0.100$; data not shown).

3.2.3. Exploratory behaviors

There was no main effect of treatment for any of the exploratory behaviors (all $ps > 0.071$), and no interaction treatment * condition ($ps > 0.286$).

3.2.4. Female prosocial behavior

There was no main effect of treatment on the frequency of resting with another female ($F_{3,34} = 1.33$, $p = 0.281$, $\eta^2 = 0.11$) nor with a male ($F_{3,34} = 1.60$, $p = 0.207$, $\eta^2 = 0.12$). The interaction treatment * experimental condition was, however, significant with regard to resting with a male ($F_{15170} = 2.99$, $p < 0.001$, $\eta_p^2 = 0.21$). This prompted tests for simple main effects of treatment within each of the experimental conditions. It turned out that there was a treatment effect during exposure to chocolate. The Tukey HSD test revealed that the females treated with PPT rested less with males than the females treated with oil ($p = 0.006$, $d = 2.13$). There was no effect at any other experimental condition. Data are shown in Fig. 9.

None of the treatments affected the frequency of sniffing another female or of the males. Likewise, the interactions between treatment and experimental condition was nonsignificant (all $ps > 0.605$; data not shown).

3.2.5. Female antisocial behavior towards males and females

The frequency of nose-off or of fleeing involving other females or males was not affected by treatment and there was no interaction between treatment and experimental condition ($ps > 0.075$; data not shown).

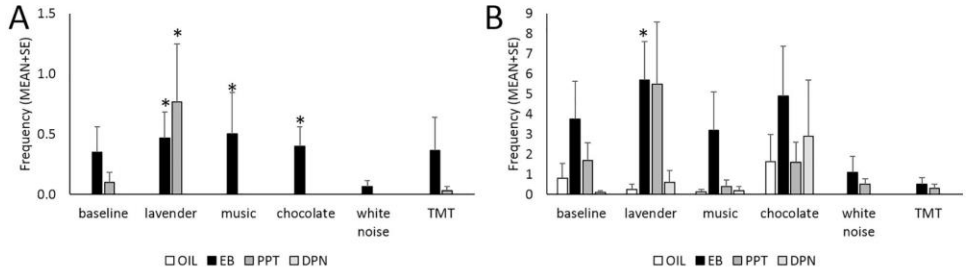

Fig. 8. Effect of the treatment on female attractivity to males. A. Mount frequency. B. Pursuit frequency. Kruskal-Wallis test, post hoc: Conover test. Data are mean ± SEM. *, different from the Oil group. Oil: n = 8, EB: n = 10, PPT: n = 10, DPN: n = 10.

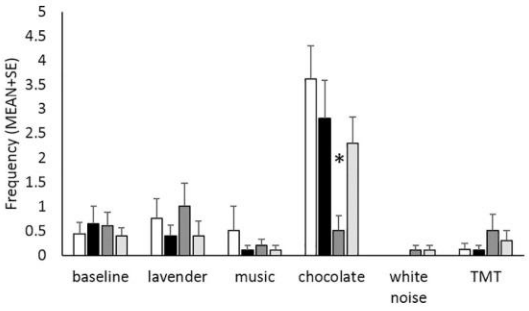

Fig. 9. Effect of the interaction between the treatment and the experimental condition on the frequency of female resting with a male. Repeated measures two-way ANOVA, post hoc: Tukey HSD. *, different from the Oil group. Oil: n = 8, EB: n = 10, PPT: n = 10, DPN: n = 10.

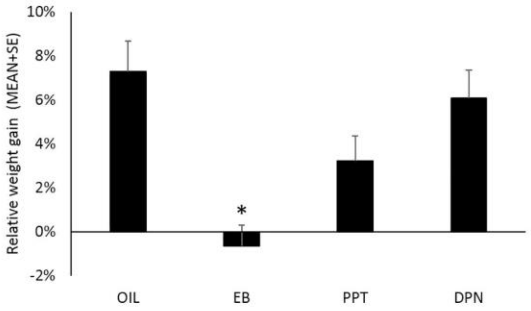

Fig. 11. Effect of the treatment on weight gain. Repeated measures two-way ANOVA, post hoc: Tukey HSD. Data are mean ± SEM. *, different from the Oil group. Oil: n = 8, EB: n = 10, PPT: n = 10, DPN: n = 10.

3.2.6. Female non-social behavior

There was no main effect of treatment on drinking, resting alone and self-grooming and there was no interaction between treatment and experimental condition (all *ps* > 0.230; data not shown).

3.2.7. Treatment effects on white-noise specific behaviors

Data for all the condition specific behaviors, except "hide alone" and "hide with another rat", greatly deviated from the normal distribution and were analyzed with non-parametric statistics. There was no treatment effect on the frequency of hiding alone, hiding with another rat, freezing, or startle (all *ps* > 0.662). The proportion of females fleeing from the noise differed between the treatments ($\chi^2_3 = 8.43$, $p = 0.038$; V = 0.47) and so did the latency to flee ($H_{3, N=38} = 8.23$, $p = 0.041$, $\eta_H^2 = 0.22$). More females in the PPT group fled from the noise (Fisher exact test, $p = 0.038$, d = 0.66; Fig. 10A) and they had a shorter latency to flee than the Oil group ($p = 0.008$, $\delta = 0.50$; Fig. 10B). None of the other treatments differed from oil (*ps* > 0.512).

3.2.8. Treatment effects on chocolate-specific behaviors

The treatments did not influence the frequency of grabbing a chocolate pellet, of eating or sniffing the chocolate or the latency to approach the chocolate (all ps > 0.210; data not shown).

3.2.9. Weight gain

We calculated the weight difference between the moment the females were introduced into the seminatural environment and the moment they were removed. The weight change was expressed as a proportion of initial weight. After the 8 days spent in the environment, the weight gain was not evenly distributed among the females ($F_{3,34} = 9.62$, $p < 0.001$ $\eta_p^2 = 0.46$). The EB group gained less weight than the Oil group ($p < 0.001$, $d = 2.36$). The PPT and the DPN groups did not differ from the Oil group (PPT-Oil: $p = 0.068$, $d = 1.22$; DPN-Oil: $p = 0.852$, $d = 0.36$). Data are shown in Fig. 11.

3.2.10. Summary of the treatment effects

EB, and to some extent PPT, stimulated the sexual behaviors. These treatments also enhanced some aspects of females' attractivity. DPN had no effect. Pro- or antisocial behaviors were not modified by any of the treatments. This was also the case for exploratory behaviors.

EB stimulated sexual behaviors at baseline, during exposure to lavender odor and music, and when chocolate was available. During white noise or fox odor, EB-treated females did not differ from those treated with oil. PPT enhanced these behaviors only during exposure to lavender odor. During exposure to lavender odor and music, as well as when chocolate was available, EB enhanced female attractivity. PPT did so only during exposure to lavender odor. When chocolate was available, PPT reduced the time spent resting with males, and during white noise this compound facilitated the flight reaction. DPN did not affect any behavior in any condition.

3.3. Analysis of co-occurrences

Co-occurrence analysis identified the behavioral associations typical of the experimental condition without considering treatment. The baseline condition showed modest associations between sniffing another rat, nose off and fleeing. In a common cluster, we find white noise and chocolate availability. Associated to the white noise we find rearing. The main behavior in this cluster is sniffing the floor, probably a result of the enhanced exploratory behavior observed during these two conditions. Lavender odor is associated with the sexual behaviors

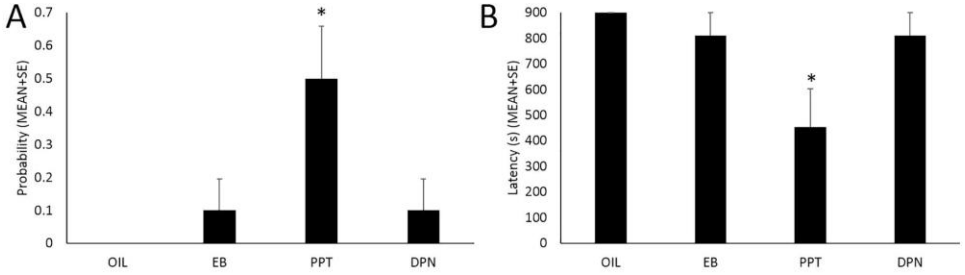

Fig. 10. Effect of the treatment on the response to the onset of white noise. A. Probability for an individual to flee from the white noise; Fisher exact test; statistical significance: *, different from the Oil group. Latency to flee the noise at its onset (B); Kruskal-Wallis test, post hoc: Conover test; statistical significance: * different from the Oil group. Oil: n = 8, EB: n = 10, PPT: n = 10, DPN: n = 10.

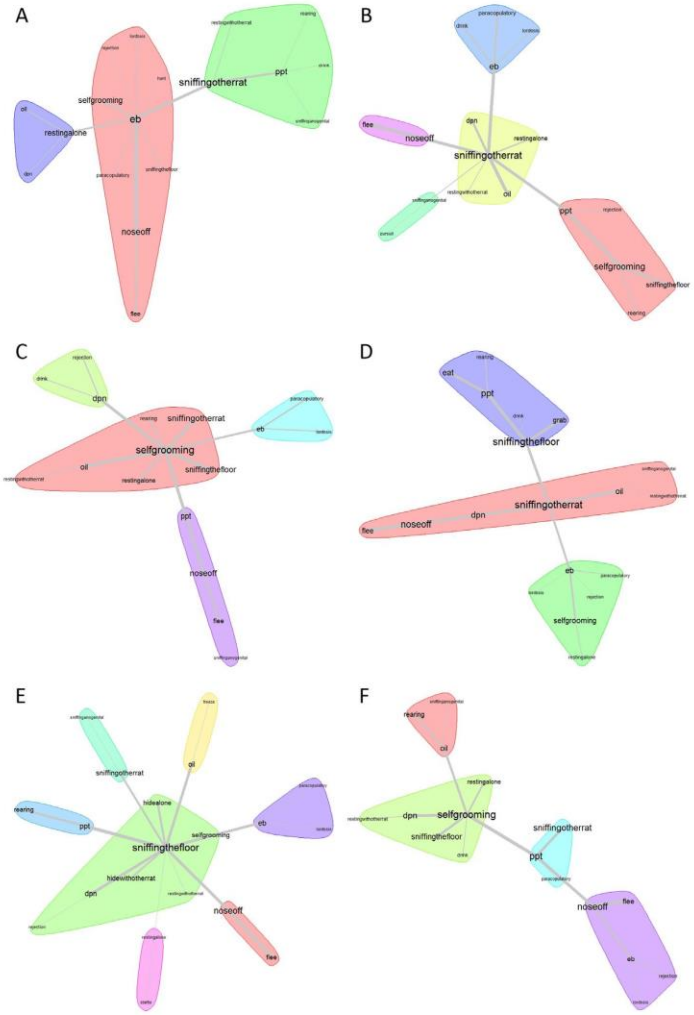

Fig. 12. Co-occurrence analysis showing main behavioral associations typical of each of the treatments. A. Baseline. B. Exposure to lavender odor. C. Music. D. Chocolate. E. White noise. F. Fox (TMT) odor. Clusters of behavioral association are represented in halos of different colors.

as well as with male pursuit of the female, anogenital sniffing, and rejection. Finally, there is a cluster containing the conditions of fox odor and music, and the behaviors of resting alone and resting with another rat. Perhaps this illustrates that these conditions somewhat reduced social behaviors, making the non-social activity of resting more pre-eminent.

Analysis of treatment effects, ignoring experimental conditions, revealed that EB and PPT each belonged to a different cluster whereas Oil and DPN were found in the same cluster. EB is associated with the sexual behaviors and drinking, whereas PPT is associated with exploratory behaviors, grooming, chocolate related behaviors and behavior patterns indicative of fear like freezing, hiding and startle. Oil and DPN are mainly related to sniffing other rats and nose off.

We then evaluated the treatments in each experimental condition. During baseline, Oil and DPN were found in the same cluster, whereas EB and PPT were in separate clusters (Fig. 12A). During exposure to lavender odor, Oil and DPN were found in the same cluster, while EB was clearly associated with sexual behaviors, and PPT was related to exploratory behaviors and grooming. Data are found in Fig. 12B. During music, a separate cluster containing EB and sexual behavior was found. PPT was found in another cluster, together with nose-off and fleeing. DPN formed a cluster together with rejection and drinking, whereas Oil was associated with exploratory and social behaviors (Fig. 12C). Chocolate exposure again made Oil and DPN appear in the same cluster, with EB and PPT clearly separated. EB was associated with sexual behaviors, whereas PPT had a strong association with chocolate-related and exploratory behaviors (Fig. 12D). White noise seemed to have altered behavior. All treatments now belonged to different clusters, and some behavior patterns formed clusters unrelated to the treatments. However, behaviors associated with white noise avoidance were found in the same cluster as DPN (Fig. 12E). Fox odor also caused each of the treatments to belong to different clusters, with EB having a minor

association with lordosis, and PPT with paracopulatory behavior (Fig. 12F). DPN appeared in a separate cluster during exposure to TMT.

4. Discussion

4.1. General considerations

The data obtained in this study are meaningful only if two requirements are met: That the emotion-inducing procedures actually induced the intended emotion and that the experimental treatments were active with about the same intensity during the 5.83 h between the beginning of the baseline observation and the end of the observation during fox odor exposure. If one or both of the requirements fail, then we cannot determine the effects of negative and positive emotions on female sexual behavior. Likewise, it would be impossible to determine the role of the estrogen receptors in the responses to the experimental conditions.

Concerning the effectiveness of the experimental conditions, it is evident that some of them indeed altered behavior in the expected way. This is particularly the case for chocolate availability and white noise. The data show that the availability of chocolate had clear behavioral effects. Both prosocial, antisocial, and exploratory behaviors were stimulated. All these responses may be interpreted as manifestations of increased arousal. It is unlikely that the enhanced arousal was caused by a fear reaction, since the females made more visits to the open area than at baseline. It is known that food reward causes increased arousal (Killeen et al., 1978), often called food arousal (Tuersley and McCrohan, 1987), in addition to positive affect. The response to chocolate observed here is, then, what would be expected if this stimulus indeed induced positive affect. White noise clearly caused a fear reaction, manifested as avoidance of the open area and enhanced antisocial behavior. The effect of lavender odor on non-sexual behaviors was limited to enhanced activity in the open area. Whether this is a manifestation of positive affect, as we expected during lavender exposure, is an open question. However, the fact that lavender odor altered sexual behavior reinforces the notion that this stimulus might have had the desired effect. To the contrary, music did not produce any behavioral manifestation of positive affect. It rather appeared to cause a fear reaction, since the time in the open area was reduced. Finally, fox odor had very slight behavioral effects, and it cannot be concluded that the females responded with fear to this odor. It is worth noting, though, that the co-occurrence analysis localized music and fox odor to the same cluster. Perhaps recent data showing that TMT is inferior to fox feces for producing fear responses in rats (Rampin et al., 2018) could explain the modest effect of this compound. This proposal is, of course, at variance to a substantial number of reports on the effectiveness of TMT (reviewed in Rosen et al., 2015).

In sum, the data allow us to suggest that chocolate availability, white noise, and perhaps lavender odor, had the intended effects, whereas music rather seemed to have an effect opposite to what we expected. Fox odor had slight effects, especially when compared to the other aversive stimulus, white noise. Nevertheless, we conclude that the first requirement mentioned above is, at least partially, satisfied.

The second requirement, the constant effect of the treatments during the entire observation period, is only possible to answer for oil and EB. The no-effect of oil is obviously constant, and there are good reasons to believe that the effects of EB outlast the observation period. We have earlier reported that female sociosexual behaviors are remarkably stable during the entire period of behavioral estrus in intact, cycling females in a seminatural environment. There is no significant change in lordosis frequency or LQ from the moment the first lordosis of the estrus period is displayed until the last lordosis (Chu and Ågmo, 2014). The change from non-receptivity to full receptivity and vice versa is almost instantaneous (Chu and Ågmo, 2015a). Similar data have been obtained in ovariectomized females, given the same doses of EB and P as used here. The duration of behavioral estrus in those females was 6.35 ± 0.42 h (mean ± SEM) (Le Moëne et al., 2015). This is longer than the duration of the present observation. Thus, the intrinsic effects of EB should have remained constant under all experimental conditions. The duration of the effects of PPT and DPN is unknown, but there is no compelling reason to assume that it is much different from that of EB. The same molecular events underlying sexual behavior are probably activated both by EB and PPT, and once activated these events will have a similar time course (see Pfaff, 2017 for an extensive review of the molecular events underlying estrogen-induction of lordosis). Other actions, as well as those of DPN, can have a different time course, so present data need to be interpreted with some caution. Nevertheless, we propose that the effects of the treatments remained reasonably stable throughout the observation period.

In addition to the two requirements discussed above, there are to caveats to the meaningfulness of the data obtained in this experiment. The first is the possibility that one or several of the experimental conditions influenced the subsequent condition or conditions. We have no data to refute this possibility. However, it can also be argued that a sequence of events probably is part of rats' nightly experience in their natural habitat. Consequently, our design would contribute to enhance external validity compared to an experiment consisting of a single event. Nevertheless, it cannot be excluded that the specific sequence used here somewhat affected the results.

A second caveat concerns the confounding effects of potential circadian rhythms causing variations in behavior during the rather long observation. This, however, is highly unlikely. There is no change in receptivity from the beginning to the end of estrus (Chu and Ågmo, 2014). Locomotor activity shows peaks at both ends of the dark period, but it remains at a stable, high level during the middle part (Spiteri et al., 2012). Also food intake remains stable during that period (e.g. Kersten et al., 1980). Thus, circadian variations cannot explain the differences between the experimental conditions. Finally we would like to point out that the males' sexual activity was as high at the end of the observation period as at baseline (mean ± SEM number of mounts was 0.38 ± 0.18 at baseline vs. 0.31 ± 0.22 during exposure to TMT, $V = 36$, $p = 0.412$).

4.2. Negative and positive emotions and sexual behavior

One aim of the present study was to determine the effects of fear on female sexual behavior. White noise strongly inhibited sexual behaviors. In fact, females treated with EB did not show more of these behaviors in the presence of the noise than females treated with oil. It is interesting to note that fox odor also eliminated the difference between oil- and EB-treated females, even though this odor had no observable effects on behaviors indicating fear or anxiety. Perhaps female sexual behaviors are more sensitive to potential threats than other behaviors, at least in the seminatural environment. Little is known about the differential effect of stress on the entire behavioral repertoire, and the relative sensitivity of each behavior to the environmental context. It could even be proposed that observations of sex behavior in the seminatural environment are a potential, externally valid procedure for evaluating anxiolytic and anxiogenic drugs. This possibility should be further explored. We also want to point out that this is the first study in which the effects of aversive or fearful stimulation present in the test situation on sexual behavior have been evaluated.

While fear-inducing situations inhibited female sexual behavior, it appears that situations putatively leading to positive affect enhance these behaviors. This is evident for lavender odor, in which both EB- and PPT-treated females showed a non-significant tendency to display more sexual behaviors than in at baseline. These treatments also enhanced female attractivity to the males during the presence of lavender odor. Considering that lavender odor might induce a state of positive affect, it could be suggested that such affect facilitates female sexual behaviors, and makes the female more attractive to males. Whether the enhanced female attractiveness was due to factors intrinsic to the

female or to lavender-induced, enhanced male responsivity to the females is not known. However, since female receptivity also was increased during exposure to lavender odor, it is likely that lavender-induced changes in the females was the main factor behind the observed behavioral changes. Chocolate availability did not have any particular effect on the sexual behaviors, despite the fact that its consumption should have caused positive affect, just as lavender odor. The fact that our observations were limited to the moment when chocolate was available may, however, obscure any possible effect. The behavioral consequences of the positive affect caused by chocolate availability might have been counteracted by the urge to collect and consume the pellets.

Too little is known about the actions of lavender odor and chocolate to make any informed speculation about the causes of differences in effects on female behavior. Furthermore, as was the case with aversive stimuli, the influence of positive affect on sexual behavior has not been studied before, rendering any effort to propose explanations for these discrepancies still more difficult.

The many studies of the effects on female sexual behavior of drugs producing positive or negative affect (e.g. Guarraci and Bolton, 2014; Ågmo, 2014) are not directly relevant for the issue of how emotional state might alter sexual behaviors. The drugs have many effects in addition to altering the emotional state (e.g. Paredes and Ågmo, 2004; López, 2010), making such studies difficult to interpret. In fact, drug effects are usually explained in terms of altered neurotransmission rather than in terms of altered emotional states.

4.3. Anxiogenic and anxiolytic effects of estrogen receptor activation

We did not obtain much evidence for estrogen effects on fear behavior. EB and DPN had no effect whatsoever on the frequency of individual behavioral items, whereas PPT showed two signs of having produced or enhanced fear reactions. It reduced female resting with males in the presence of chocolate, and it enhanced the flight reaction in the presence of white noise. Both these effects can be interpreted as manifestations of fear or anxiety. PPT would then be anxiogenic in the chocolate and noise conditions. These conditions were associated with heightened arousal, and it has been shown that PPT indeed is anxiogenic in such situations (Lund et al., 2005; Morgan et al., 2004; Spiteri et al., 2010a; Spiteri et al., 2010b; see also Borrow and Handa, 2017, for a review). It is also noteworthy that PPT belonged to a cluster separate from the other treatment clusters at all experimental conditions. This shows that the females treated with this compound had a behavioral structure different from all other treatments. This fact can probably be attributed to the fact that PPT stimulated sexual behaviors under some conditions and enhanced anxiety-like behaviors under others.

The complete lack of effect of DPN on the frequency and duration of the behaviors recorded here would indicate that the ERβ receptor is of little or no importance for sexual activity as well as for fear and anxiety in test procedures with external validity. However, the co-occurrence analysis showed that DPN belonged to a separate cluster in the situations that might be considered aversive, i.e. during exposure to music, white noise and fox odor. In the neutral or positive conditions, i.e. at baseline, during exposure to lavender odor and chocolate, DPN and oil belonged to the same cluster. This important observation suggests that actions at the ERβ only becomes apparent in contexts being aversive or even inducing fear. It appears, then, that present data confirm the lack of a role for the ERβ in sexual behavior as well as its importance for anxiety-related behaviors.

The potential role of the membrane receptor GPER1 has not been mentioned. This receptor is obviously activated in the EB-treated females, and perhaps also in the females treated with PPT. High concentrations of this ERα agonist bind to the GPER1, and DPN might be still less active (Petrie et al., 2013). Since the GPER1 has been implicated in fear responses as well as in female sexual behavior (reviewed in Hadjimarkou and Vasudevan, 2018), and since there is evidence for crosstalk between GPER1 and the ERα, it is possible that the GPER1 may have contributed to the effects observed in the present study. This issue is, however, too complex for being analyzed here.

Another issue not addressed here is the possible contribution of local synthesis of estrogens. Although such synthesis has been suggested to affect some of the behaviors studied here (reviewed in Cornil, 2018), present data have no relevance for this question.

4.4. On the utility of a seminatural environment and the problem of opposing effects of ERα and ERβ

When the experimental subjects are given the opportunity to express a substantial proportion of their natural behavioral repertoire, the multitude of data generated needs to be made comprehensible in some way or another. Moreover, the frequency or duration of behavioral items give only a rudimentary description of behavior. Behavior patterns are displayed in a continuous flow, and the sequence of behavior is completely ignored in frequency and duration analysis. The co-occurrence analysis and the clustering and visualization techniques employed here makes the patterning of behavior intelligible, and subtle modifications can be discovered. This, for example, made it possible to see that DPN affected behavior in aversive situations, even though the frequency and duration of none of the behaviors was altered.

At the time of the experimental manipulations, the rats had lived in the seminatural environment for 7 days. Consequently, they have had plenty of time to familiarize themselves to the environment and to the other rats. One manifestation of this is the almost complete absence of aggressive interactions. It is reasonable to assume that the subjects considered the environment as a safe place. We introduced the experimental conditions upon this baseline. A similar approach was employed in the studies of fear and aggression in the visual burrow system (Blanchard et al., 1995; Blanchard et al., 2001; Blanchard and Blanchard, 1989), a procedure not entirely different from the one used here. The present results may be most illustrative with regard to the behavioral consequences of the activation of the estrogen receptors.

In nature, rats live and copulate in groups, and most of their activities are localized within the well-known home range. These characteristics are preserved in the seminatural environment but entirely absent in most other tests. It can be maintained that our procedure satisfies the requirements for a representative design in brunswikian terms (see Brunswik, 1955; Petrinovich, 1980, also Chu and Ågmo, 2016). Data from such designs have external validity in the sense that they might be applicable to situations other than the same in which the data were obtained. Therefore, we propose that activation of the ERα in female rats leads to the display of sexual behaviors and enhanced fear in unsafe or novel situations, even outside the laboratory setting. The ERβ does not modify the sexual behaviors, but it may be important for reducing fear in fear-inducing contexts, even outside of the laboratory setting.

Unfortunately, opposing actions of the ERα and ERβ would complicate the understanding of the behavioral actions of estrogens. In the intact animal, both receptors would be stimulated simultaneously, and opposing actions would then be nulled out. It is difficult to find a situation in which circulating estradiol would stimulate one receptors and not the other, meaning that opposing actions would not be physiologically relevant. The solution to this conundrum is not immediately apparent.

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5. Silencing ERs in specific brain sites

5.1 Introduction

The role of ERs and their respective implication in socio-sexual and fear-related behaviors may result from regional differences in ER expression in brain regions modulating distinct behavior patterns. Indeed, silencing ERs site-specifically offers a much finer approach than agonistic treatment or knock-out models. These experimental designs give valuable insight into the ERs' role at the organism level but fail to express that estrogen-dependent behavioral responses result from (1) situation-dependent activation of functionally distinct brain areas, (2) ERs' distribution in these areas.

5.2 Site-specific regulation of estrogen-dependent behavioral responses

To date, very few studies have used a shRNA encoded with an adeno-associated virus (AAV) to site-specifically knock-down ERs in the female brain. So far, these studies confirmed that ERα in the VMN supports all aspects of female sexual behavior, from lordosis reflex (Snoeren et al., 2015; Spiteri et al., 2010b), paracopulatory behaviors (Spiteri et al., 2010b, 2010a), interest in intact males (Spiteri et al., 2010b) and attractivity to males (Snoeren et al., 2015). This receptor showed no role in sexual behavior in the bed nucleus of the stria terminalis or the medial amygdala (Snoeren et al., 2015). In the pre-optic area, ERα stimulated female attractivity to males, and social investigation (Snoeren et al., 2015). Finally, in the medial preoptic area ERα increased locomotor activity in familiar environments, and movement velocity in threatening ones (Spiteri et al., 2012). So far, only one study has analyzed the effects of ERβ in the medial pre-optic area and the medial amygdala, however, this study focused on male behavior (Nakata et al., 2016). Therefore, the effects of ERβ in different brain structures in females remains in the land of the unknown.

The differential activation of brain structures depending on the situation might be a key factor in understanding the actions of ERs. In Paper III, I investigated the differential role of ERs in the VMN, a structure essential to the activation of sexual behaviors, and the CeA, a brain area involved in immediate fear reactions. These structures show differential distribution of ERs (Fig. 5), which could correlate the relative importance of each receptor in the functions governed by these brain areas.

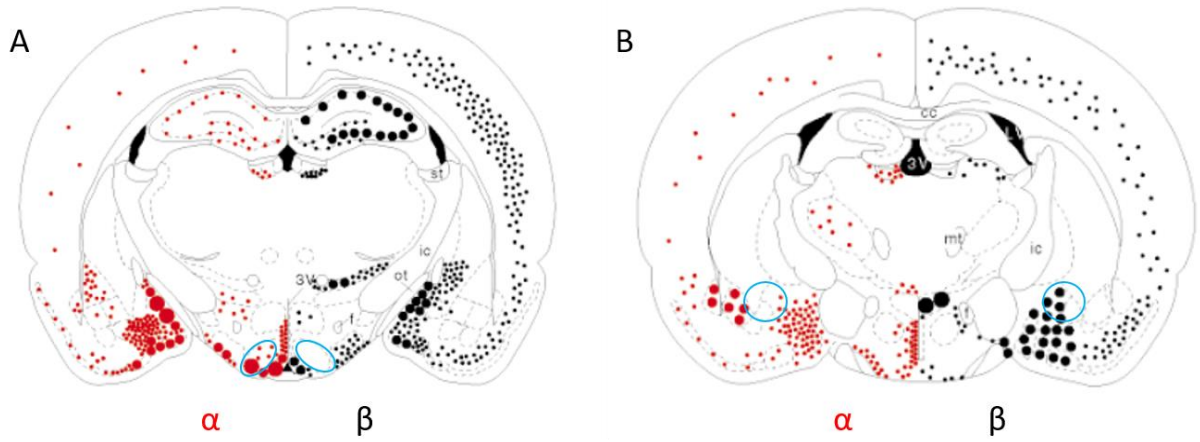

Fig. 5. Schematic representation of coronal sections depicting the distribution of ERα (left side; red dots) and ERβ (right side; black dots) mRNA in the rat brain. Small dots = 1–5 labeled cells; medium dots = 56–10 labeled cells; large dots = approximately 50 labeled cells. A. Ventromedial nucleus of the hypothalamus (blue circle), antero-posterior -3.14 mm. B. Central amygdala (blue circle), antero-posterior -1.80 mm. Adapted with permission from Shughrue et al., 1997.

5.3 Results of Paper III

5.3.1 Contribution of ERs in the CeA

Silencing ERα in the CeA showed no effect. However, silencing ERβ in the CeA increased risk assessment duration and decreased the frequency of food eating, both behaviors

being consistent with a pattern of increased anxiety. However, females treated with AAV against ERα and AAV against ERβ appeared in the same cluster exclusively during exposure to white noise, potentially due to the highly aversive nature of white noise. Following exposure to white noise, infusion in the CeA did not modify behavioral recovery.

In parallel, silencing ERβ also increased olfactory exploration of the seminatural environment (sniffing the floor), in particular in a positive context (lavender odor exposure). Co-occurrence analysis showed that females whose ERβ was knocked-down were associated with risk assessment and sniffing the nozzles during most stimuli, and exploration of the chocolate during this stimulus. Females lacking ERβ in the CeA therefore seemed to express higher arousal in adequate contexts.

Since silencing ERα did not modify indicators of anxiety nor arousal, but silencing ERβ elevated them. It is possible that ERβ has anxiolytic properties in the CeA, and might down-modulate arousal levels.

5.3.2 Contribution of ERs in the VMN

As expected, silencing ERα in the VMN reduced sexual behaviors, mainly the probability to display lordosis, and females lacking ERα were consistently associated with rejection of the males. LQ remained unchanged, as previously found in the seminatural environment (Snoeren et al., 2015). This treatment also suppressed the occurrence of huddling during the aversive white noise. This behavior, also recognized as “hiding with another rat”, has been associated previously with social buffering in fear-inducing conditions. Since silencing ERα suppressed social buffering associated with fearful situations, it is possible that ERα is anxiogenic.

In the analysis of co-occurrence, during exposure to white noise females infused with AAV against ERα in the VMN were associated with rearing an exploratory behavior mostly expressed in safe contexts (Oloruntobi et al., 2014). This seems to confirm the anxiogenic properties of ERα in the VMN, in accordance with a previous report (Morgan et al., 2004). Silencing of ERβ in the VMN showed no direct effect on behavior, independently from the environmental conditions.

Following exposure to white noise, female infused in the VMN recovered exploration of the open area within 350 s following white noise offset. Females infused with AAV against ERα did not display huddling behavior in the 50 seconds following white noise offset while control females still did. Disruption of ERα expression seemed to advance recovery from white noise, compared to control females.

5.4 Conclusions

In the VMN, expression of ERα was necessary to lordosis display. This receptor in the VMN also convey anxiogenic properties. I found no role for ERβ in this brain area. In the CeA, ERα did not modify behavioral responses, but ERβ showed several anxiolytic effects. This possible double dissociation further reinforces the need for site-specific knock-down studies in order to resolve the issue of opposite ER actions by understanding their differential contribution in distinct brain areas.

Paper III

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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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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Conflicts of interest: None declared

Highlights:

- Estrogen receptor α is essential for female sexual behaviors.
- Activation of estrogen receptor β in the central amygdala reduces anxiety.
- Estrogen receptor β modulates arousal level.
- The use of a seminatural environment highlights the context-dependent role of ERs.

Abstract

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 x 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 x 120 x 80 cm, l x w x 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 lux during the day and 30 lux 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. 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 minutes). 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 (35g) (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 am 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 am the females were captured and injected with EB. On day 7 at 9.00 am 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 minutes 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).

[Table1]

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 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/mm^2) 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 seminatural 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 were 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 minutes 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).

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 prosocial 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 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).

[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).

3.3.3 Effect of treatment on exploratory behavior

Treatment in the CeA influenced olfactory exploration of the seminatural 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 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 most fear-related behaviors (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 most noise-specific behaviors (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).

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).

3.5.3 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 nose-off. AAV-ERβ formed a distinct cluster with various behaviors: sniffing the floor, eating food 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 minutes 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 synthetized. 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].

4.4 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.

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Figure captions:

Figure 1. (A) Picture of the seminatural environment. (B) Drawing of the seminatural environment and division in zones.

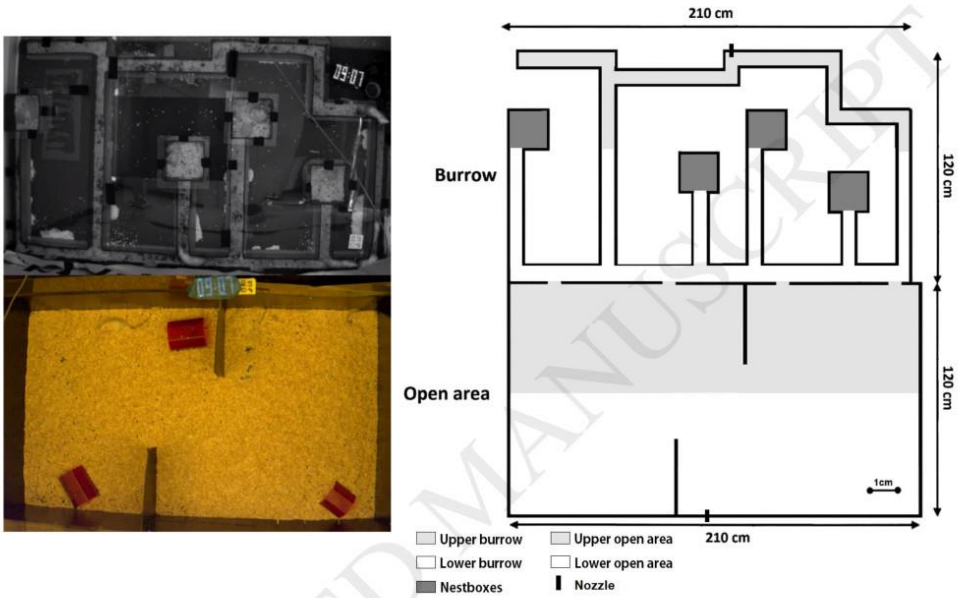

Figure 2. Infusion sites within the central amygdala (CeA; Panel A) and the ventromedial nucleus of the hypothalamus (VMN; Panel B) with shRNA directed against the ERα (AAV-ERα; in red), ERβ (AAV-ERβ; in blue) or luciferase (AAV-luc; in black). Numbers to the right represent distance (in mm) from bregma.

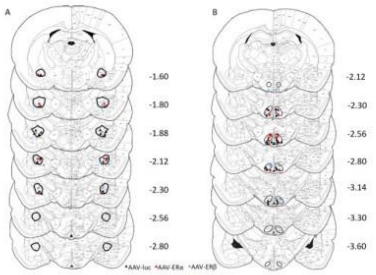

Figure 3. Immunocytochemical staining of estrogen receptor expression in brain slices in the CeA and the VMN after bilateral infusion of (A) AAV-ERα or (B) AAV-ERβ. The panels to the far left show pictures from control females treated with AAV-luc. The figures to the right show the density of ERs. Data are mean+SEM. *, different from AAV-luc.

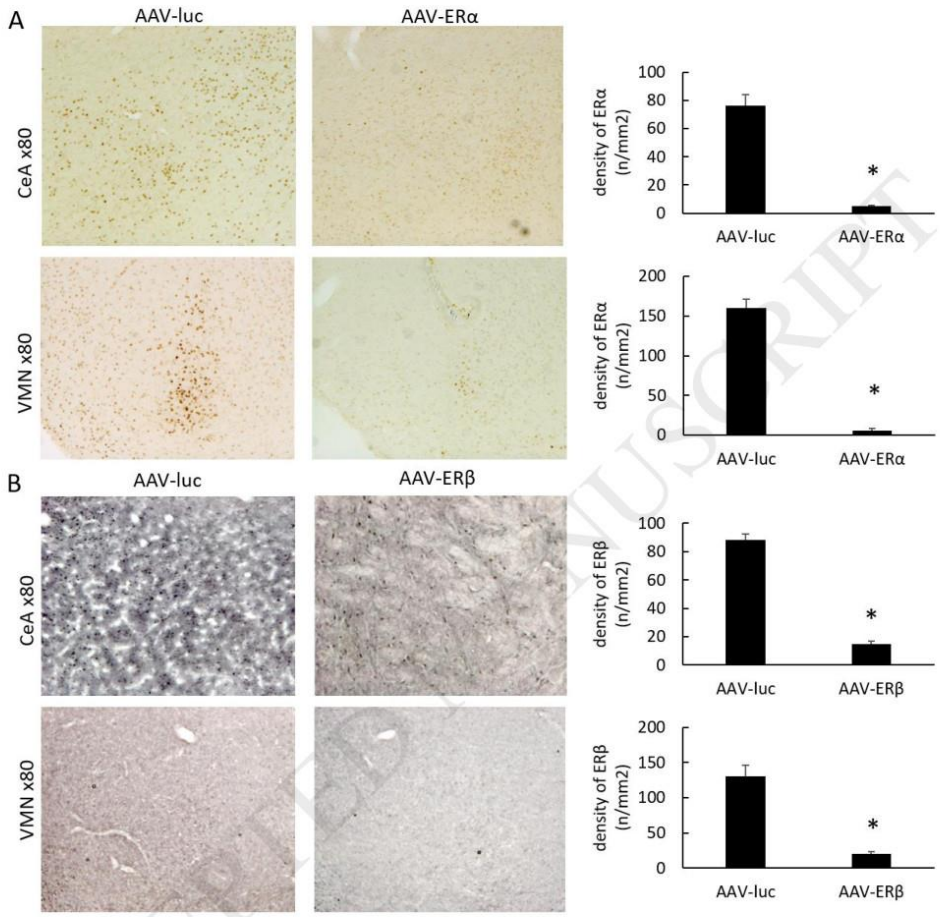

Figure 4. (A) Proportion of females infused with shRNA directed against the ERα or the ERβ in the VMN displaying lordosis, all emotion-inducing stimuli collapsed; (B) Lordosis frequency in these females, all emotion-inducing stimuli collapsed, mean+SEM; (C) Proportion of females displaying lordosis at each of the emotion-inducing stimuli. *, different from AAV-luc.

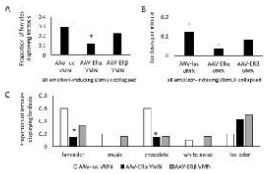

Figure 5. (A) Frequency and duration of sniffing the floor in females infused with shRNA directed against the ERα or the ERβ in the CeA, all emotion-inducing stimuli collapsed; (B) Duration of sniffing the floor in these females at each of the emotion-inducing stimuli, data are mean+SEM. *, different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

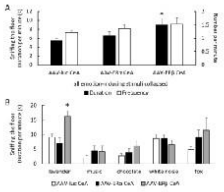

Figure 6. (A) Frequency and duration of risk assessment in females infused with shRNA directed against the ERα or the ERβ in the CeA, all emotion-inducing stimuli collapsed; (B) Frequency and duration of huddling during exposure to white noise, in females infused with shRNA directed against the ERα or 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.

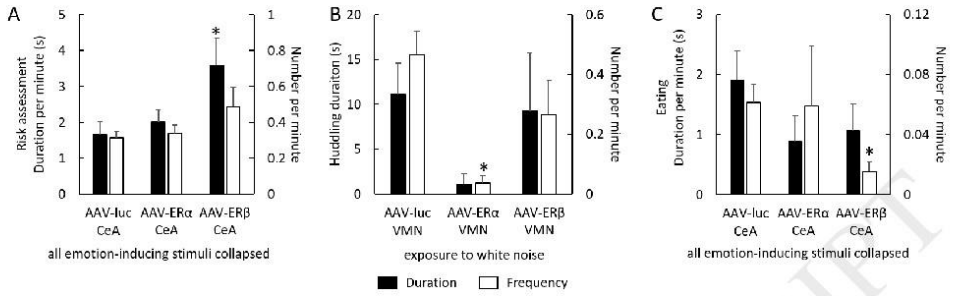

Figure 7. Co-occurrence analysis showing main behavioral associations typical of each of the treatments in the CeA (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ), during exposure to (A) lavender odor, (B) music, (C) chocolate, (D) white noise and (E) fox odor (TMT). Clusters of behavioral 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.

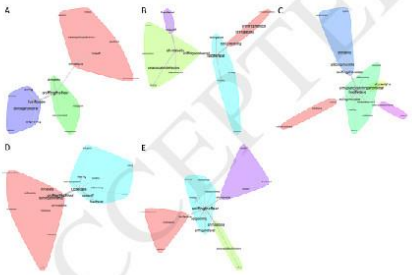

Figure 8. Co-occurrence analysis showing main behavioral associations typical of each of the treatments in the VMN (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ), during exposure to (A) lavender odor, (B) music, (C) chocolate, (D) white noise and (E) fox odor (TMT). Clusters of behavioral 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.

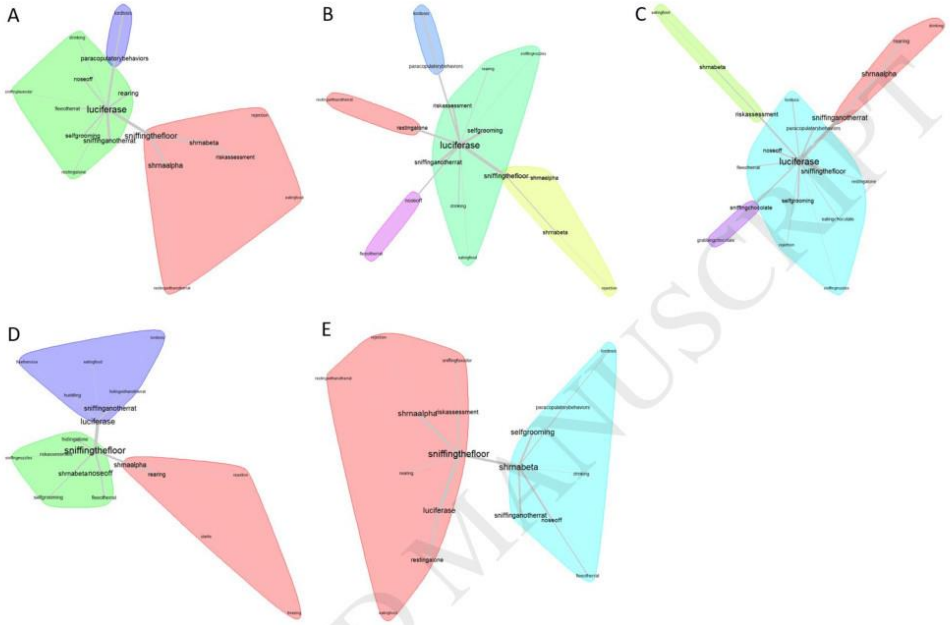

Figure 9. (A) Frequency and duration of huddling during the 450 s following white noise offset in females infused with shRNA directed against the ERα or the ERβ in the VMN; *, different from AAV-luc. Huddling frequency (B), Huddling duration (C), Frequency and duration of sniffing the floor (D), Time spent in the open area (E), from the last 50-seconds of white noise exposure to 450 seconds 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 seconds preceding it. Thus, 0 shows the behavior between -50 and 0 seconds 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.

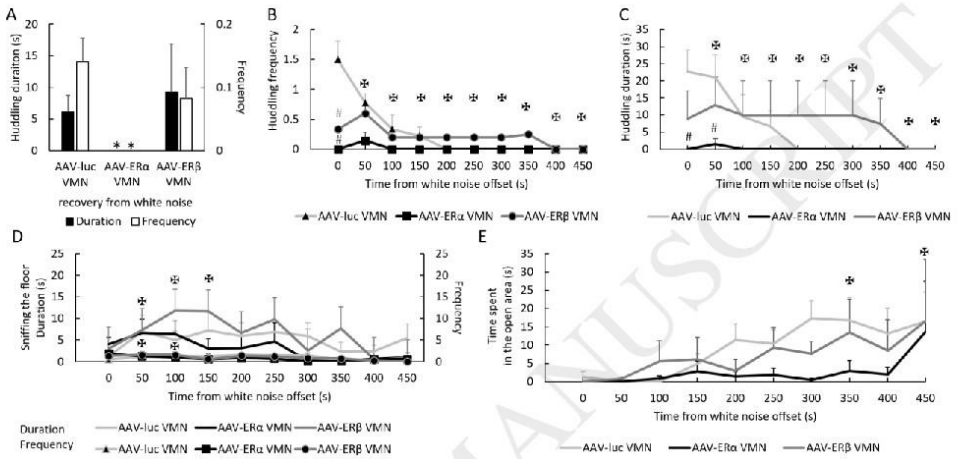

Figure 10. Co-occurrence analysis showing main behavioral associations typical of each of the treatments (luciferase, AAV-luc; shrnalpha, AAV-ERα; shrnabeta, AAV-ERβ) during the 450 s following the exposure to white noise, (A) CeA and (B) VMN. Clusters of behavioral association are represented in halos 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.

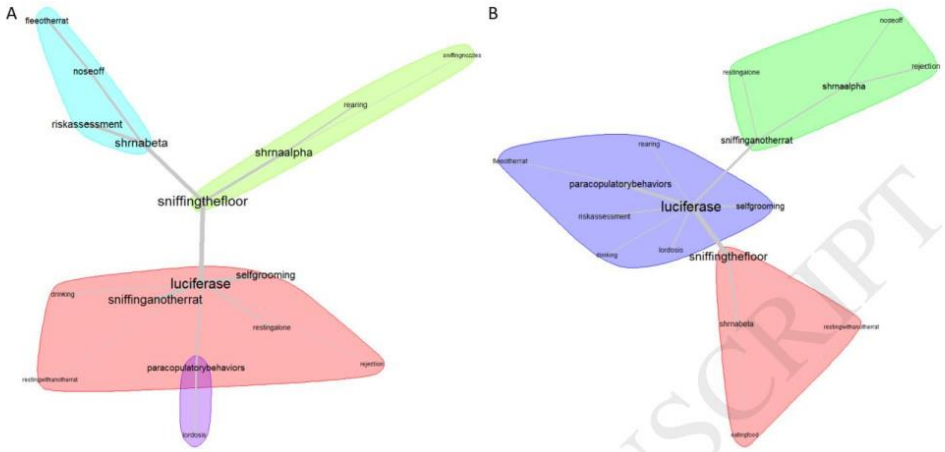

Table 1. Ethogram, definition of recorded behaviors. f = frequency; d = duration; l = latency; o = occurrence. [a], behavior observed only in the presence of chocolate. [b], behavior only observed during exposure to white noise.

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.
Female attractivity	Rejection; f	Female kicks, boxes or assumes a belly up posture.
	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

Exploratory behaviors and ambulatory activity		female.
	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	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 chocolate[a]; f,d	Chew 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	Rush 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	Approaching 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.

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	Resting alone	-0.10±0.21	+0.76±0.33	-0.05±0.15	***-1.00±0.00****	***+0.87±0.27****

maintenance behaviors	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

6. General discussion

This thesis aimed at determining the role of ERα and ERβ, at the organism level and specifically in the VMN and the CeA, in a procedure with external validity. In a seminatural environment, behavioral reactions are buffered by several factors, notably perceived controllability and social buffering (Paper I). Despite these phenomena, sustained behavioral changes are observable. Therefore, I propose that such observations are especially robust and transferrable.

6.1 Estrogen receptors and sexual behaviors

In accordance with previous reports (Mazzucco et al., 2008; Ogawa et al., 1998; Rissman et al., 1997), I found that ERα agonist was necessary for the display of lordosis and paracopulatory behaviors, and that this receptor enhanced female attractivity to males (male mount and pursuit of the females) (Paper II). ERβ failed to activate female sexual behaviors regardless of the context, confirming that ERβ is not involved in these behaviors.

Knock-down of ERα in the VMN was sufficient to disrupt lordosis display (Paper III), consistently with previous findings (Snoeren et al., 2015; Spiteri et al., 2010b). Knock-down of this receptor in the CeA did not affect female sexual behavior, confirming that this brain area in not involved in these behaviors. Consistently with my previous findings using an ERβ agonist, knock-down of ERβ, whether in the CeA or the VMN had no influence on sexual behavior.

In the adult female rat, expression of ERα in the VMN was essential to the display of sexual behavior. ERα also enhanced female sexual motivation and attractivity to males. ERβ was not involved in sexual behaviors.

6.2 Estrogen receptors and social behaviors

In my experiments, the only effect of ERα on social behavior was to reduce the frequency of resting with males, but not with females, during exposure to chocolate pellets in females treated with an ERα agonist (Paper II). Therefore, I propose that my result is merely coincidental to the increase in chocolate-related activity observed in females treated with this agonist. Apart from this result, treatment with ER agonists did not produced any effect on pro- or antisocial behaviors, contrarily to studies showing some involvement of ERs in social behavior, at least in aggression (Ogawa et al., 1998, 1996). More specifically, silencing ERs in the VMN or the CeA did not modify pro- nor anti-social behaviors (Paper III).

An earlier experiment proposed that differences in aggression in females lacking ERα resulted from the context of exposure, and that the effects of ERα only appeared in a novel, unfamiliar test cage, but not when the females were tested in their familiar home cage. Thus, the absence of an effect of ERα on antisocial behaviors in the seminatural environment would be consistent the rats' familiarity with their surroundings, considering that they had been living in the seminatural environment for several days at the time of the behavioral observations. In addition, most studies of aggression used the resident-intruder test (Ogawa et al., 1998; Spiteri et al., 2010a), whereas in the seminatural environment, all rats were familiar to each other, which might account for the stability of social behaviors observed.

In established groups of rats living in a familiar environment, ERs seemed to play no role in the regulation of social interactions. Specifically, neither ER expression in the VMN nor in the CeA showed any involvement in these behaviors.

6.3 Estrogen receptors and anxiety- and fear-related behaviors

At the onset of white noise, yohimbine-treated females had a significantly shorter latency to flee the noise at its onset (Paper I). By comparison, females treated with ERα agonist had a higher probability to flee the noise (Paper II). These somewhat similar results seemed to indicate that ERα is anxiogenic to a certain extent. This is consistent with previous report of anxiogenic properties of ERα agonist in fear-inducing procedures (Lund et al., 2005; Spiteri et al., 2010a). Reduction in the number of ERα in the VMN suppressed social buffering during exposure to white noise (Paper III). This last effect further supports that ERα has anxiogenic properties in this brain structure. Nevertheless, this receptor showed no role in fear- and anxiety-related behaviors in the CeA, an area involved in the regulation of these responses. This might be due to the low expression of ERα in the CeA (Österlund et al., 1998; Shughrue et al., 1997; Shughrue and Merchenthaler, 2001), or to parallel processing of different kind of threats by different brain structures (Canteras et al., 2012).

Even though systematic administration of ERβ agonist did not modify behavior quantitatively, it modified behavioral structure during exposure to aversive stimuli. Then, in accordance with other reports, ERβ properties were apparent in response to fearful situations (Kudwa et al., 2014; Walf et al., 2008; Walf et al., 2009). During exposure to aversive stimuli, females treated with ERβ agonist were associated with behaviors of self-maintenance, suggesting lower anxiety levels (Paper II). In addition, following knock-down of ERβ in the CeA, I observed increased risk assessment and decreased eating (Paper III). These modifications are consistent with increased anxiety. Therefore, the anxiolytic properties of ERβ seem to be, at least partly, modulated through in the CeA.

Finally, females having a reduced number of ERβ showed increased display of sniffing the floor during exposure to lavender odor and were associated with chocolate-related behaviors during this stimulus. Both lavender odor and chocolate were attractive to the rats and did not seem to present any aversive properties (Paper I, Paper II). Therefore, in the absence of fear, it is difficult to formulate that higher interaction with these stimuli derived from ERβ anxiolytic effects. The most parsimonious conclusion would be that, in females, a reduction in the number of ERβ in the CeA could contribute to increased arousal in response to attractive stimuli.

The results of Paper III suggests that a finer understanding of ERs' role within different brain structures could resolve the issue of seemingly opposite ERs' roles that has concerned a large part of the literature investigating estrogen actions since the identification of a second receptor.

Estradiol acting through ERα had anxiogenic properties in response to aversive white noise, notably in the VMN. The anxiolytic effects of estradiol appeared to be attributable to the ERβ, partly through its actions in the CeA, regardless of the emotional stimulus induced.

7. Future directions

The role for G protein-coupled estrogen receptor 1 (GPER1) has not been addressed here. It is very well possible that this compound might have accounted for some of the results reported here. Non-genomic estrogen effects would substantially complicate the present results. Thus, I focused exclusively on the effects of ERα and ERβ on behavior in different environmental contexts, but it should be acknowledged that GPER1, or local estradiol synthesis, may also influence behavioral responses.

The externally valid procedure used in this thesis highlighted important context-dependent changes that shed new light to behavioral modifications. For example, modification of locomotor activity during a fearful situation depended on the availability of a shelter zone. Moreover, this thesis identified areas needing further research. Notably, more comprehensive studies of the ERs' role on rat sociality are lacking. Such studies would benefit from including unknown vs familiar conspecifics in a safe home cage or a novel test cage. Overall, externally valid procedures might allow to overcome the reductionist bias that has prevailed in modern neuroscience.

This thesis highlighted the heterogeneous contribution of ERs in brain areas modulating different functions. The use of shRNA encoded with adeno-associated viral vectors selectively silencing one of the two identified ERs will greatly improve our knowledge of estrogen actions on arousal and anxiety levels, as well as on exploratory, social and sexual behaviors. With insight into the role of each receptor within different brain areas, production of typical behavior depends on the balance between activation of ERα and ERβ. Inadequate expression or activation of one or the other isoform could account for several of the sex differences in neurodevelopmental and neurodegenerative disorders.

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