Role of estrogen alpha receptors in sociosexual behavior in female rats housed in a seminatural environment.

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[f] Sergei Musatov was killed in a tragic accident on May 27, 2015. This paper is dedicated to his memory

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Abstract

This study investigated the role of estrogen receptors alpha (ERα) in the ventromedial nucleus of the hypothalamus (VMN),the preoptic area (POA), the medial amygdala (MePD) and the bed nucleus of stria terminalis (BNST) in sociosexual behavior in female rats. This was done in two sets of experiments, with the VMN and POA investigated in the first set, and the MePD and BNST in the second set. The VMN and POA received intense projections from the MePD and BNST.

We used a short hairpin ribonucleic acid (shRNA) encoded within an adeno-associated viral (AAV) vector directed against the ERα gene to reduce the number of ERα in the VMN or POA (First set of experiments), or in BNST or MePD (second set of experiments) in female rats. The rats were housed in groups of four ovariectomized females and three males in a seminatural environment for 8 days. In comparison to traditional test set-ups, the seminatural environment provides an arena in which the rats can express their full behavioral repertoire, which allowed us to investigate multiple aspects of social and sexual behavior in groups of rats. Behavioral observation was performed after estrogen and progesterone injections.

A reduction of ERα expression in the VMN or POA diminished the display of paracopulatory behaviors and lordosis responses compared to controls, while the lordosis quotient remained unaffected. This suggests that ERα in the VMN and POA play an important role in intrinsic sexual motivation. The reduction in ERα did not affect the social behavior of the females, but the males sniffed and pursued the females with reduced ERα less than the controls. This suggests that the ERα in the VMN and POA is involved in the regulation of sexual attractiveness of females. The ERα in the MePD and BNST, on the other hand, plays no role in sociosexual behavior.

Introduction

Sexual behavior in female rats is highly dependent on ovarian hormones. Estrogens act via two different estrogen receptors, the estrogen receptor α (ERα) and the estrogen receptor β (ERβ). Studies in both rats and mice have shown that the ERα is important for the activation of sexual behaviors (1-5). The ERβ, on the other hand, is not necessary to induce receptivity (5). In addition to the role in sexual behaviors, estrogens also affect other types of behavior such as general arousal (6), fear and anxiety (2, 7), social recognition (7, 8), object memory (9), and aggression (7, 10).

One of the main sites of action of estrogen is the ventromedial nucleus of the hypothalamus (VMN). The role of the VMN in female sexual behavior has been studied extensively. Lesions of the VMN result in dramatic decreases in lordosis and paracopulatory behaviors (11), while electrical stimulation results in a facilitation of lordosis (12). The ERα plays an important role in the VMN, since infusion of short hairpin ribonucleic acid (shRNA) encoded within an adeno-associated viral (AAV) vector directed against the ERα gene into the VMN reduced sexual receptivity and paracopulatory behaviors in rats and mice (1, 13). In addition, local infusions of antiestrogens in the VMN decrease lordosis in rats (14).

Another important area for estrogen effects is the preoptic area (POA). POA lesions have been shown to abolish paracopulatory behavior, while promoting the lordosis reflex (15). This indicates that the POA plays a dual role in sexual behavior in females. Single-cell recordings showed that different subsets of neurons in the POA are involved in the regulation of the different behaviors (16). Lesions of the POA also decrease the preference for intact male rats, suggesting that the POA plays a stimulatory role in sexual motivation (17). The role of the ERα in the POA, however, is rather confusing. A reduction of ERα caused by shRNA infusions into the POA resulted in increased levels of lordosis responses, while paracopulatory behaviors remained unaffected (2). This suggests that ERα could play a role in

the inhibitory function of the POA in lordosis, but not in the regulation of paracopulatory behaviors (2). Interestingly, AAV-ERα-POA females also showed reduced preference for an intact over a castrated male (2).

The VMN and POA receive intensive neural inputs from the medial posterodorsal amygdala (MePD) and the bed nucleus of the stria terminalis (BNST) (18-20), which in turn receives projections both from the main and accessory olfactory systems. It is thought that the olfactory stimulation will reach the VMN and POA via the amygdala (18, 19), mainly through the BNST. Olfactory stimuli are crucial for the activation of approach behaviors (21, 22), and without approach copulation will never occur.

Lesion studies have shown that the MePD reduces approach behavior of sexually receptive females towards male rats (23). The ERα in the MePD, however, do not seem to play a role in the regulation of this behavior in particular, since a reduction in ERα did not affect the approach towards male rats (1). Therefore, the BNST might play an essential role in the regulation of approach behavior. Similar to the VMN, POA, and MePD, the BNST contains a high number of ERα (24, 25), suggesting that if this brain area is involved in approach behavior, this might act via ERα.

A reduction in ERα in the MePD, in addition, did not affect approach behavior in female rats, but did reduce the total time spent in the incentive zones of both the intact and castrated males (1). Therefore, Spiteri et al. suggested that a reduction of ERα in the MePD might also affect social motivation, since both stimulus rats also have social incentive properties (1). The current knowledge of the role of ERα in social behaviors is rather limited. We only know that estrogens increase social recognition (26, 27), which might be regulated via the ERα in the MePD (7). As reviewed by Yamamuro (28), social behavior is normally investigated in a social interaction test in which the time spent in social interaction (sniffing or grooming each other) between pairs of unfamiliar rats in neutral arenas is evaluated.

Unfortunately, traditional test set-ups have limited amount of space and time for rats to interact with each other. In addition, the use of only pairs of rats limits the opportunity to explore social interaction and does not model the natural situation in which rats live in groups (29, 30).

The same constraints occur in sexual behavior testing. In nature, rats copulate in groups consisting of one or several estrus females and several males (29, 30). Interestingly, observational studies performed in seminatural environments have revealed that the mating patterns in groups of rats are quite different from the mating patterns observed in the traditional laboratory mating tests with pairs of rats or mice (31-37). It is therefore essential to adjust the study design for this type of research. In the current study, we investigated the role of the ERα in social and sexual behavior in rats. Therefore, we needed to develop a paradigm in which the rats were able to express their full repertoire of behaviors. The use of a seminatural environment circumvents this limitation and provides the opportunity to investigate the social and sexual behavior in groups of rats. The difference in test set-up also allows making observations in a situation in which the animals can freely escape from sociosexual situations instead of being forced to interact (as in the traditional smaller set-up). This study, therefore, uses the seminatural environment to investigate the role of ERα in the VMN, POA, MePD, and BNST in sociosexual behavior in rats. In the first set of experiments, the ERα in the VMN and POA were investigated, while the ERα in the more upstream regions (the MePD and BNST) were studied in the second set of experiments.

Materials and methods

Subjects

In total sixty-four female and forty-eight male Wistar rats (200-250 grams at the start of the experiments) were obtained from Charles River (Sulzfeld, Germany). Half of the rats

were used in POA and VMN studies and half in MePD and BNST studies. The rats were housed in same sex pairs in Macrolon IV® cages (60x38x20 cm) on a reversed 12 hours light/dark cycle (lights off between 11 am and 11 pm), in a room with controlled temperature (21±1 °C) and relative humidity (55±10%). Standard rodent food (RM1, Special Diets Services, Witham, Essex, UK) and tap water were available ad libitum. All experimentation was conducted in agreement with the European Union council directive 86/609/EEC and approved by the National Animal Research Authority in Norway.

Stereotaxic surgery,infusion of the viral vector, and ovariectomy

Surgery was performed under ketamine/xylazine anesthesia (100 mg/kg and 10 mg/kg, respectively). The females were fixed in a stereotaxic frame and two small holes were drilled in the skull to allow lowering of cannulas bilaterally (homemade of stainless steel with a diameter of 30 gauge, and a length depending on the target areas) to the appropriate positions. Coordinates according to the Paxinos and Watson atlas (38) were: POA: anteroposterior -0.4 mm, mediolateral ±0.5 mm, dorsoventral -8 mm; VMN: anteroposterior -2.56 mm, mediolateral ±0.5 mm, dorsoventral -9.6 mm; BNST: anteroposterior -0.92 mm, mediolateral ±1.5 mm, dorsoventral -6.6 mm; MePD: anteroposterior -3.1 mm, mediolateral ±3.6 mm, dorsoventral -7.8 mm below the dura. These coordinates were verified in another pilot study that was performed before the start of the present study. In this pilot, several females (for each structure) were euthanized with an overdose of pentobarbital, and cannula were placed at the intended location. One µl of methylene blue was infused bilaterally through the cannula. The brain was then removed and immediately frozen sectioned for determinations of the infusion site.

In the experimental subjects, one microliter PBS containing 10^9 genomic particles was infused per cannula. In each set of experiments, twenty-two females received an AAV vector

encoding for short hairpin ribonucleic acid (shRNA) against the ERα gene (AAV-ERα) (11 females per target brain area) and another 10 were injected with an AAV vector expressing a shRNA directed against the luciferase gene (AAV-luc). In each set of experiments, 2 brain areas were targeted; the VMN and POA in the first, and the MePD and BNST in the second. Since previous studies have shown that AAV-luc is essentially inert with regard to the ERα receptor, we divided the 10 rats and injected 5 with AAV-luc in one brain area and 5 in the other. In the statistical analysis, the AAV-luc rats were combined, giving a reasonablu large control group.

shRNA is an artificial RNA molecule with a tight hairpin turn that can be used to silence target gene expression via RNA interference (RNAi), with a relatively low rate of degradation and turnover. Both vectors (AAV-luc and AAV- ERα) contain, in addition, an independent enhanced green fluorescent protein (EGFP) expression cassette under the control of a hybrid CMV/chicken beta-actin promotor. The AAV-luc was used to control for any potential nonspecific adverse effects of surgery or toxicity of encoded products and the EGFP was used as a reporter to visualize transduced neurons. A detailed description of the vector, including the nucleotide sequences, can be found in (13). The infusion was performed over a period of 10 minutes after which the cannulas were left in place for another 5 minutes. After cannula withdrawal, the skin was sutured with surgical clips. Then, the subjects were immediately ovariectomized during the same anesthesia. The ovariectomy enables the possibility to regulate the females' sexual receptivity with hormone injections.

After the surgery, the subjects were left undisturbed for 3 weeks in order to allow the AAV-ERα to fully express and the number of ERα is reduced. The permanent reduction in ER is specific to the ERα, and does not affect the ERβ (13). Furthermore, diffusion of the viral vector is limited to a small area around the infusion site. It has been reported before that

infusion of the vector into the VMN does not affect an adjacent structure like the arcuate nucleus (1), for example.

All males used in this experiment were intact. They, therefore, were not submit to any surgery or hormonal treatment.

Hormone treatment

The female rats were taken out of the seminatural environment just before they received a subcutaneous hormone injection. This happened on day 5 and 7 of the experiment. On day 5, the ovariectomized females received 18 μg/kg estradiol benzoate (EB), and on day 7 1 mg progesterone (P). All injections took place at 9:30 am, after which the females were immediately placed back in the seminatural environment at the same locations as they were caught. The males stayed in the environment during this procedure, since the males received no hormonal treatment.

EB and P (Sigma, St. Louis, MO, USA) were dissolved in peanut oil (Apoteksproduksjon, Oslo, Norway), and injected in a volume of 1 ml/kg.

Apparatus

In both experiments, the rats were tested in a seminatural environment (2.8 x 2.4 x 0.75 m) consisting of two parts, a burrow system and an open area, connected with 4 small openings (8 x 8 cm). A firmly fitted, thick, black cloth was used to divide the experimental room in two, so that the burrow area could be sealed off from the open area. This made it possible to vary light intensity in the open area while maintaining complete darkness in the burrow. (More details and a drawing of this environment can be found in (35)) In the open area, a lamp 2.5 m above the center provided a light of 180 lx from 11pm to 11am. During the night (11am to 11 pm), the light intensity was reduced to about 1 lx, approximately equivalent

to the light provided by a full moon. A similar lamp in the burrow area provided 24 hours of 1 lx light, so that the light in the burrow was always reduced to full moon. Two video cameras (Sanyo VCC-6592P) equipped with a zoom lens (Computar T6Z5710-CS 5.7 – 34.2 mm) were installed in the ceiling, one above the burrow and one above the open field. The cameras were connected to hard disk drive DVD recorders (Sony RDR-HX780) with a capacity large enough to record 72 h of video of good quality. Every 72 h the contents on the hard disk were transferred to DVDs for storage.

Procedure

The rats were housed in groups in the seminatural environment for 8 days. A group of rats consisted of 4 female and 3 male rats, which were unfamiliar with each other and sexually inexperienced. Before each group was introduced, the floor in the open area, tunnels and nest boxes was covered with about 2 cm of aspen wood chips (Tapvei, Harjumaa, Estonia). About 2 kg of food pellets were put on the floor, close to a corner in the open area. Twelve aspen wood sticks, 2 x 2 cm, 10 cm long (Tapvei, Harjumaa, Estonia) were randomly distributed in the open area, and 3 red polycarbonate huts (15 x 16.5 cm, height 8.5 cm; Datesend, Manchester, England) were irregularly placed closed to the middle. In addition, 6 pieces of a small square mat of non-woven hemp fibers (5 x 5 cm, 0.5 cm thick; Happi mat, Datesend, Manchester, England) were put in each nest box in the burrow area.

In order to distinguish between the rats on the video record, a rectangle, about 2 x 3 cm, was carefully shaved on the back of the rats the day before introduction into the environment. One female had the rectangle close to the tail, another in the middle of the back, and a third had it close to the neck. The fourth female was not marked on the back. In addition, the tail was marked with one, two, or three transversal, thick black lines. The fourth

female was not marked. Males were marked exactly as the females but the tail marks were made larger to distinguish between males and females.

All experimental groups were introduced into the environment around 1 pm (day 0), and the video recorders were activated. Recording was continuous until the end of the observation period (day 8). After the observation period, the subjects were removed and the environment was carefully cleaned and disinfected before the next experimental group was introduced.

Behavior analysis

The videos from each group were transferred from the DVDs to an external hard disk. Observations were made with the Observer XT, version 10 (Noldus, Wageningen, the Netherlands) software. Although, the complete 8 days were originally, recorded, only the video record from 1:15-2:15 pm on day 7 of the experiments was examined for this experiment. This observation time was chosen because ovariectomized females are most receptive 4 hours after the progesterone injection (39). The descriptions of the scored behaviors are listed in Table 1.

The behavioral analysis of this study was performed on day 7, because pilot studies have revealed that it takes 4-5 days before the exploratory behavior of the rats in such an environment reach stable levels. The rats should be familiar with the environment before the sexual behavior was investigated. Therefore, the hormone injections started at day 5 and 7 resulting in sexual receptivity on day 7.

Design

Two sets of experiments were performed in this study. In the first set, the role of the ERα in the VMN and POA were investigated, while in the second set, the ERα in the MePD

and BNST were studied. Therefore, AAV-ERα and AAV-luc treated female targeted in the VMN or POA were used in set one, while AAV-ERα and AAV-luc treated female targeted in the BNST or MePD were used in set two. In each set of experiments, 8 groups of rats were placed in the seminatural environment, each group consisting of 4 females and 3 males. So, in total 32 females and 24 males were used per experiment. The AAV-ERα and AAV-luc treated females were randomly divided between and within the groups. This meant that in one group of rats, both AAV-ERα and AAV-luc treated females were available (a description of the composition of each group is listed in Table 2). When introduced to the environment, the rats were unfamiliar with each other and sexually naïve.

Immunocytochemistry

The day following the last experiment, the subjects were euthanized with an overdose of pentobarbital. They were perfused with PBS followed by 4% formaldehyde. The brain was removed and postfixed overnight at 4°C in 4% formaldehyde. It was then rinsed with PBS and cryoprotected in 30% sucrose in PBS. After 24 hours in the sucrose solution, the brain was frozen in isopentane cooled on dry ice, and then transferred to a -80°C for storage until processing.

The brains were frozen-sectioned at 50 μm with a sledge microtome. Sections containing the target areas were collected and processed according to a conventional free-floating protocol. Antibodies against the ERα (Rabbit polyclonal to ERα, 1:25,000; Millipore, Upstate, NY, USA) and EGFP (Goat polyclonal to GFP, 1:5000, Abcam, Cambridge, UK) were used in combination with secondary antibodies (2.5 μl/ml biotinylated rabbit and goat, respectively; Vector Laboratories Inc., Burlingame, CA, USA) and avidin-biotin peroxidase complex (ABC Elite Kit from Jackson Immunoresearch, West Grove, PA, USA) to identify cells containing ERα and transduced cells, respectively. After antibody reactions, sections

were stained with diaminobenzidine. This staining gave a brown coloration to EGFP. Hence, neurons transduced with the viral vector were labeled by brown cytoplasmic staining. In contrast, the ERα is colored purple. Thus, brown-stained cells indicated injection localization while purple-stained cell showed ERα expression. For comparison between experimental and control groups, the number of ERα-stained cells in the target areas was determined. For this, photomicrographs were taken with a Zeiss Axiophot photomicroscope (Carl Zeiss, Obercochen, Germany) connected to a digital camera (Nikon DS) and appropriate software (Camera Control Unit DS-L1). Then, the pictures were transferred to a computer and opened in Photoshop software. With the help of photoshop, a zone was drawn (always of the same size and at the same location) over the target areas. On the photomicrographs, we counted manually all the stained cells inside the zone. This was done for 2-5 slices per animal, from which the average was used for further calculations. The average of the counted number of ERα was divided by the surface in order to obtain a density (number of ERα/mm^2) that was used for the analysis.

Data analysis

The behavioral data from the open and burrow area were analyzed separately. A Shapiro-Wilk test showed no homogeneity of variance. All behavioral data was, therefore, analyzed using the non-parametric Kruskall-Wallis test, followed by Mann Whitney-*U* correction post hoc testing. A two-tailed significance level of 0.05 was used in all tests. (A different type of data analysis in which all same-treatment rats in each group in the seminatural environment is used as an experimental unit can be found in the supplementary information. There was no important change in results.)

For the histological data, an independent sample *t*-test was used to determine the differences in ERα expression in the targeted brain areas. Again, a two-tailed significance level of 0.05 was used.

Results

ERα in the VMN and POA

Histology

Due to unexpected intense background staining during one batch of slices processed for ICC, we were unable to obtain a sufficient receptor count on 18 of 32 brains. However, the immunocytochemistry on the other 14 brains revealed that these females were infused correctly into the intended nuclei. Both in the VMN and in the POA, the number of ERα was significantly reduced with ca. 73% and 63%, respectively, after infusion with AAV-ERα compared to AAV-luc (VMN: $t(6)=4.323$, $p=0.005$, POA: $t(4)=11.288$, $p<0.001$) (Figure 2).

Sexual behavior

All sexual activity took place in the open area. As shown in Figure 3, a reduction in the number of ERα caused a decrease in the number of paracopulatory behaviors performed by the females. While no significance was found in the AAV-ERα-POA females, AAV-ERα-VMN females showed significantly less paracopulatory behaviors compared to AAV-luc females ($z=-2.564$, $p=0.010$) (Figure 3a). Both the AAV-ERα-POA and the AAV-ERα-VMN females showed significantly less lordosis responses than the AAV-luc (VMN: $z=-2.498$, $p=0.012$; POA: $z=-2.167$, $p=0.030$) (Figure 3b). The reduction in lordosis responses is probably caused by a decrease in received mounts (VMN: $z=-2.431$, $p=0.015$; POA: $z=-1.836$, $p=0.066$) and intromissions (VMN: $z=-2.560$, $p=0.010$; POA: $z=-2.433$, $p=0.015$) of the AAV-ERα-VMN and AAV-ERα-POA females compared to control females (Figure 3c),

because there was no difference in lordosis quotient (Figure 3d). This means that most mounts and intromissions were accompanied by a lordosis response.

Social behavior performed by the females

No significant differences were found on the amount of social behaviors performed by the AAV-luc and AAV-ERα-POA and AAV-ERα-VMN females in the burrow area. Although, the females with reduced numbers of ERα in the POA or VMN sniffed other rats less often than the control females, this effect was not significant. No differences were found in the burrow area on the other social behaviors like amount of grooming others, pursuing, fighting and nose-off behavior (Table 3).

In the open area, on the other hand, both the AAV-ERα-POA and AAV-ERα-VMN females approached other rats significantly less often than the AAV-luc females (POA: z=-1.889, p=0.05; VMN: z=-2.916, p=0.004). The AAV-ERα-POA females also significantly kicked less often other rats than the AAV-luc (z=-2.266, p=0.023). This effect was not seen in AAV-ERα-VMN females. The reduction in ERα in the POA or VMN did not affect any others social behaviors in the open area (Table 3).

Social behavior performed by the males

The male rats pursued both, the AAV-ERα-VMN and AAV-ERα-POA females, shorter (VMN: z=-2.209, p=0.027; POA: z=-1.879, p=0.060) and less often (VMN: z=-2.323, p=0.020, POA: z=-1.918, p=0.05) than the control females in the open area (Figure 4ab). In the burrow area, a similar effect was found on the time pursuing the different females (VMN: z=-2.427, p=0.015; POA: z=-1.879, p=0.06) and the number of pursues (VMN: z=-2.392, p=0.017; POA: z=-1.918, p=0.05) by the males. In addition, the males sniffed the AAV-luc females significantly longer than the AAV-ERα-VMN females (z=-2.543, p=0.011) in the

open area, an effect that was not found for the AAV-ERα-POA females. In the burrow area, no differences on the time sniffing females were found. The males also sniffed the AAV-ERα females as often as the control females in both the burrow and open area (Figure 4cd).

The males confronted the AAV-ERα treated females less with fights than the AAV-luc females. Both the AAV-ERα-VMN and the AAV-ERα-POA females (VMN: $z=-2.264$, $p=0.024$; POA: $z=-2.169$, $p=0.03$) were being fought a significantly shorter time in the burrow area than the control females (Figure 4e). This effect was not seen though in the number of fights they received (Figure 4f).

As shown in Table 3, no differences were found on any other social behaviors the males performed towards the females. A reduction in ERα in the POA or VMN did not affect the amount of time being anogenitally sniffed, groomed or nosed-off in the open and burrow area. Although the males seem to approach control female more regularly than the AAV-ERα females, this effect was not significant.

ERα in the MePD and BNST

Histology

The immunocytochemistry on all 32 brains revealed that all females were infused correctly into the intended nuclei. However, the infusion of AAV-ERα failed to reduce the number of ERα receptors in three rats (one in the AAV-ERα-MePD and two in the AAV-ERα-BNST). These rats were removed from further analysis (though, it should be mentioned that the exclusion of these cases did not change the outcome of the experiment). In the other 29 brains, it was found that the number of ERα was significantly reduced to ca. 70% (MePD) and 75% (BNST) after infusion with AAV-ERα compared to AAV-luc (MePD: $t(11)=8.830$, $p<0.001$, BNST: $t(8)=7.189$, $p<0.001$) (Figure 5).

Sexual behavior

No significant differences were found between the AAV-ERα-BNST, AAV-ERα-MePD and AAV-luc females in sexual behaviors during the day of receptivity in the burrow area or open field. A reduction in ERα in the MePD or BNST did not affect the number of paracopulatory behaviors and lordosis responses performed by the females (Figure 6ab). In addition, no effects were found on the number of received mounts, intromissions and ejaculations, and the lordosis quotient (Figure 6cd).

Social behavior performed by the females and males

Again, no differences in the social behavior of the females were found (Table 4). The AAV-ERα-MePD and AAV-ERα-BNST females socially interacted as long and as often with other rats as AAV-luc females. In addition, the males pursued, sniffed and fought with each female in an equal manner (Figure 7).

Sexual and social behavior with separate AAV-luc-MePD and AAV-luc-BNST controls

In order to verify whether our strategy to pool the AAV-luc-MePD and AAV-luc-BNST in one control group of AAV-luc, we analyzed the same data in which we compared AAV-ERα-MePD (n=10) with AAV-luc-MePD (n=5) and AAV-ERα-BNST (n=9) with AAV-luc-BNST (n=5). Again, no differences were found between the AAV-ERα-BNST, AAV-ERα-MePD and AAV-luc-BNST and AAV-luc-MePD females, respectively, in sexual behaviors during the day of receptivity in the burrow area or open field (data not shown). In addition, no differences were found in the social behavior of the females, or the behavior performed by the males (data not shown). Therefore, we concluded that the results are identical to those obtained when the pooled control was used.

Discussion

ERα in the VMN and POA

Sexual behavior

Females with fewer ERα in the VMN or POA (first set of experiments) showed lower levels of both lordosis responses and paracopulatory behaviors compared to the control females. The reduction in lordosis responses in AAV-ERα-VMN and AAV-ERα-POA females was clearly caused by a decrease in received mounts and intromissions, since the lordosis quotient remained above 90%. Previous studies have suggested that the ERα in the VMN are essential to induce lordosis responses, but the current results suggest that this is probably not the case. Females with reduced ERα expression in the VMN or POA can still perform normal lordosis responses, although they do not show lordosis often. The reduction in lordosis responses and paracopulatory behaviors could reflect a reduction in sexual motivation. Though, it could also be suggested that few lordosis responses are caused by the remaining 27% or 37% of ERα in the VMN and/or POA, respectively.

The results of the AAV-ERα-VMN females are slightly different from previous studies using AAV-ERα to eliminate the ERα in the VMN of rats and mice (1, 13). In these studies, the reduction in ERα levels in the VMN reduced sexual receptivity and paracopulatory behaviors in rats and mice. The AAV-ERα-VMN females in our study also showed decreased levels compared to control females (1, 13). To the contrary, different results were found on lordosis quotients. While in this study the lordosis quotient remained unaffected, the other studies showed lower lordosis quotients (1, 13). In all studies, however, the number of lordosis responses is reduced.

Interestingly, the results of the AAV-ERα-POA females also differ from a previous study in rats (2). While the current study showed a reduction in paracopulatory behaviors, the other study showed no effect on this parameter in females with lower numbers of ERα in the

POA. Although AAV-ERα-POA females show less lordosis responses in this study, both studies show no effect on the lordosis quotient.

Sexual behavior in seminatural environment versus traditional test cages

One possible explanation for these differences in lordosis quotients in the VMN results could be the use of different test set-ups. The older studies have used pairs of rats placed in traditional test cages with much smaller sizes than our seminatural environment. In comparison to the traditional set-ups, the large environment allows the rats to interact with all members of the group and express their full repertoire of behaviors (35-37). The males are able to select one of the females for each sexual interaction, whereas they otherwise would be matched to only one. Besides the fact that males are able to select the most participating and receptive females, the female are at the same time able to escape from the males when they do not want sexual interaction. The current experiment shows that males only select females for mounts or intromissions that have performed paracopulatory behaviors. This is in line with another study in which intact female rats were studied in the seminatural environment (35, 40). It could, therefore, be argued that females with reduced numbers of ERα in the VMN are equally physically capable of performing lordoses as control females, but less motivated to participate in sexual interactions. The decrease in paracopulatory behaviors in a seminatural environment reflects a lower intrinsic sexual motivation (40), that in turn results in less sexual stimulations from the males. However, if the females receive occasionally a mount or intromission, they react with similar lordosis responses as control females. The AAV-ERα-VMN females tested in the small traditional copulation set-ups, on the other hand, are more or less forced to participate in the sexual interaction. The decrease in lordosis quotient could, therefore, be a result of the reduction in motivation to participate at the moments of sexual interaction.

The differences in results on paracopulatory behavior in POA targeted females, could also be explained by the use of different test set-ups. While the AAV-ERα-POA females were forced to participate in the sexual interaction in the smaller traditional set-ups, the seminatural environment allowed them to escape from this interaction. This suggests that AAV-ERα-POA females are physically capable of performing paracopulatory and lordosis behaviors when forced to sexually interact, but show reduced levels of intrinsic sexual motivation when given the choice.

In summary, the current experiment shows that the ERα in the VMN and POA play a stimulatory role in the probability female rats participate in sexual activity. Females with less ERα show reduced numbers of paracopulatory behaviors and sexual interactions, which could be explained as a decrease in intrinsic sexual motivation (40, 41). The approach behavior was also affected negatively in the AAV-ERα-VMN and AAV-ERα-POA females (Table 2). Interestingly, this conclusion is in line with the findings in the other studies performed in rats: AAV-ERα-POA and AAV-ERα-VMN females show decreased sexual motivation in the sexual incentive motivation test (1, 2).

Social behavior

As shown in Table 2, no differences were found in the amount of social behavior performed by AAV-ERα-VMN and AAV-ERα-POA females compared to controls. As reviewed in Ervin et al. 2015, estrogens are involved in many different complex social (cognitive) aspects, like social learning, social recognition, and agonistic behaviors (42). The ERα seems to be primarily involved in these behaviors, with a smaller role for the ERβ. (42) We know, for example, that estrogens increase social recognition (26, 27), but that the ERα in the VMN are not involved in the regulation (7). However, the role of ERα in basic social behavior, defined as behavioral interactions between two rats living in pairs or groups, has not

been studied previously. The current results suggest that the ERα in both the VMN and POA play no role in social behavior performed by the females themselves.

It was argued by Spiteri et al. that the lack of preference for the intact male might have been caused by an increase in social motivation (2). In the seminatural environment this could be translated in the expression of more social behaviors towards other rats. Still, these behaviors are expected to be unrelated. In fact, other prosocial behaviors, like (anogenital) sniffing of the female or grooming are completely unrelated to sexual activities (35, 40). However, our experiment suggests that the lack of preference for the intact male in the study of Spiteri et al. was not caused by an increase in social motivation.

Social behavior performed by the males

The sexual attractiveness of the females, on the other hand, seems to be affected by the reduction in number of ERα in the POA or VMN. Our experiments showed a decrease in time and frequency the males pursued the AAV-ERα-VMN and AAV-ERα-POA females. But also the amount of time spent sniffing the females with reduced ERα was decreased, although not significant for AAV-ERα-POA females. Overall, the males show more interest in the control females than the AAV-ERα females when they have the opportunity to choose between females. The lack of interest for the females with reduced ERα in the POA or VMN indicates that these female are less attractive than the control. The ERα might, therefore, be involved in the regulation of the sexual attractiveness of female rats. In males, on the other hand, it was shown in mice that the social incentive properties of ERα knockout males might be more attractive than wild type mice (43). Nevertheless, it should be mentioned that these mice were lacking all ERα, instead of the ERα specifically in one brain region.

The reduced sexual attractiveness of the AAV-ERα female rats could in theory be a direct result of the decrease in paracopulatory behaviors by the females. As mentioned before,

male rats only mount and intromit females that have shown paracopulatory behaviors (35, 40). However, the occasions in which the male starts a pursue before the female starts darting and hopping happen as often as the pursue is preceded by the female's paracopulatory behavior (40). Therefore, it is more likely that the decrease in paracopulatory behavior and the reduction in social interaction happen simultaneously, without one being a result of the other.

Indicators of intrinsic sexual motivation

The reduction in paracopulatory behaviors probably reflects lower levels of intrinsic motivation. In contrast to the number of paracopulatory behaviors itself; the intrinsic motivation state might affect sexual attractiveness. It can be hypothesized that males are able to detect the difference in levels of motivation between females. It is known that males are able to distinguish non-receptive from receptive females; they approach hormonally primed females more than non-receptive females or males in choice paradigms (44). Two examples of distant stimuli on which approach to a conspecific could depend on are olfaction or audition. Rats emit 50 kHz ultrasonic vocalizations (USVs) in the presence of a sexual partner and during copulation (45-47), suggesting that these vocalizations might signal the probability to participate in sexual interactions. Nonetheless, it was shown that the emitted USV have no incentive value for rats of the opposite sex (48, 49). The fact that females prefer devocalized males as often as other males suggest that rats do not 'communicate' their intrinsic levels of sexual motivation via ultrasonic vocalizations (50).

To the contrary, olfactory stimuli are powerful attractants. The odor of a receptive female is enough to induce approach behavior by males (48, 51-54), while anosmic males do not immediately distinguish between females in estrus and in non-estrus, and show a sustained reduction in social investigation (22). The limitation of these studies, however, is that they never investigated the attractiveness of multiple sexually receptive females. It is,

therefore, unknown whether differences in intrinsic sexual motivation of females are detectable for males in the females' odors. Though, in another study, we found that male rats show no differences in sniffing behavior towards their female of 1st choice and the other females in a partner mate choice paradigm using three sexually receptive females (55). In mice, though, male mice showed a reduced interest in the odors of ERα knockout females (56). To the contrary, the removal of ERα in only one brain area does not stop estrus in female rats. We can, therefore, assume that local ERα reduction is not sufficient to change the female's odor. We hypothesize that even though males distinguish the odor from receptive and non-receptive females, there is not any convincing reason for believing that they distinguish any possible individual differences in odor between fully receptive females (55). However, this is rather hypothetical since this issue was not addressed in the current study.

In summary, the data shows that ERα in the POA and VMN are involved in the regulation of intrinsic sexual motivation and attractiveness. Reduced expression of ERα in these brain areas result in a decrease in sexual behaviors and received social interactions. A plausible explanation for the reduction in received social interactions from the males, however, is not yet available.

ERα in the MePD and BNST

Sexual behavior

AAV-ERα-MePD and AAV-ERα-BNST females showed normal levels of sexual behavior. A reduction in ERα levels in the MePD or BNST did not affect the number of paracopulatory behaviors or lordosis responses compared to controls. Also the number of received male copulatory behaviors was similar for AAV-ERα-MePD, AAV-ERα-BNST and control females. These results are in line with a previous study that showed that the ERα in the MePD play no role in sexual behavior, investigated in a traditional mating and sexual

motivation set-up (1). Though, the MePD is involved in the regulation of sexual behavior. Lesion studies showed that the MePD plays an inhibitory role in approach behavior towards male rats (23), while enhancing lordosis intensity and paracopulatory behaviors (57, 58). However, based on our results we can conclude that ERα expression is not essential in this regulation.

The role of the BNST in female sexual behavior has not been studied extensively. Fos-immunoreactivity (Fos) studies have shown that the BNST is activated during sexual behavior (59), but the precise role of the brain area has never been investigated in rats. This study shows that the ERα in the BNST are probably not involved in the regulation of sexual behavior in female rats. This is in line with a study performed in hamsters that showed that BNST lesions did not affect male-odor preference and lordosis behavior in females (60).

Together this indicates that ERα begin to play a role in the more downstream brain areas of the regulation of sexual behavior, since the MePD and BNST project to the VMN and POA.

Social behavior

Reduced ERα expression in the MePD or BNST did also not affect social behavior in the females. Additionally, the AAV-ERα-MePD and AAV-ERα-BNST females did not receive more or less social interactions from the males than control females. This suggests that ERα in the MePD or BNST are not involved in the regulation of social behavior or sexual attractiveness.

A previous study with AAV-ERα-MePD females showed that the reduced ERα expression eliminated social recognition in rats (7). The rats in our experiment, though, were housed together for 8 days, meaning that at the day of receptivity the rats were already

familiar with each other. The lack of difference in sniffing behavior between AAV-ERα-MePD females and controls is, therefore, not surprising.

Conclusion

Overall, it can be concluded that ERα expression in the hypothalamic nuclei, the VMN and POA, is involved in intrinsic sexual motivation and attractiveness, but not in social behavior. The ERα in the MePD and BNST, on the other, plays no role in sociosexual behavior. The MePD and BNST project to the VMN and POA, suggesting that ERα begin to play a role in the more downstream brain areas of the regulation of sociosexual behavior. Olfactory stimuli are an important element in sociosexual behavior and the MePD and BNST are essential in the relay of this olfactory information to the VMN and POA. The current results suggest that ERα are not involved in the transmission of olfactory stimuli.

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