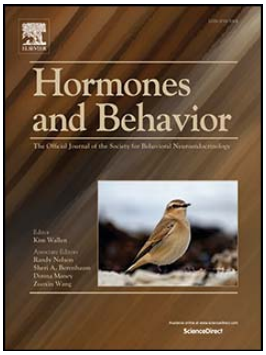

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Potential contribution of progesterone receptors to the development of sexual behavior in male and female mice

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Abstract

We previously showed that estradiol can have both defeminizing and feminizing effects on the developing mouse brain. Pre- and early postnatal estradiol defeminized the ability to show lordosis in adulthood, whereas prepubertal estradiol feminized this ability. Furthermore, we found that estradiol upregulates progesterone receptors (PR) during development, inducing both a male-and female-typical pattern of PR expression in the mouse hypothalamus. In the present study, we took advantage of a newly developed PR antagonist (ZK 137316) to determine whether PR contributes to either male- or female-typical sexual differentiation. Thus groups of male and female C57Bl/6j mice were treated with ZK 137316 or oil as control: males were treated neonatally (P0-P10), during the critical period for male sexual differentiation, and females were treated prepubertally (P15-P25), during the critical period for female sexual differentiation. In adulthood, mice were tested for sexual behavior. In males, some minor effects of neonatal ZK treatment on sexual behavior were observed: latencies to the first mount, intromission and ejaculation were decreased in neonatally ZK treated males; however, this effect disappeared by the second mating test. By contrast, female mice treated with ZK during the prepubertal period showed significantly less lordosis than OIL-treated females. Mate preferences were not affected in either males or females treated with ZK during development. Taken together, these results suggest a role for PR and thus perhaps progesterone in the development of lordosis behavior in female mice. By contrast, no obvious role for PR can be discerned in the development of male sexual behavior.

Introduction

The classic theory of mammalian brain and behavioral sexual differentiation holds that male-typical neural and behavioral characteristics develop under the influence of perinatal testosterone, whereas female-typical neural and behavioral characteristics develop in the absence of any hormonal secretions, i.e. by default. In rodents much evidence suggests that many, though not all, of the perinatal organizational actions of testosterone on the development of the male nervous system actually result from the cellular effects of estradiol formed via the local, neural aromatization of circulating testosterone (reviewed in Baum, 1979). Accordingly, it was proposed many years ago that the brain of the developing female rodent fetus is protected from the actions of maternal estradiol and/or from aromatized products of testosterone derived from male fetuses lying adjacent in the uterine horn by the relatively high affinity binding of estradiol by circulating alpha-fetoprotein (AFP) (McEwen et al., 1975). Indeed we showed that the capacity of AFP knockout (AFP-KO) female mice to display lordosis behavior in adulthood was severely attenuated and that this defect was reversed by prenatal treatment with an aromatase inhibiting drug (Bakker et al., 2006). These results provided important evidence that circulating AFP binds estradiol in female fetuses so as to protect their brains from the potential defeminizing actions of this hormone which otherwise normally occur in the male nervous system in response to estradiol synthesized from testosterone after it is taken up from the circulation.

The default organization of the female brain has however been challenged on several occasions (Toran-Allerand, 1984). Early behavioral studies showed that removal of the ovaries at birth actually reduced lordosis behavior in adult female rats, although these effects disappeared upon repeated testing (Dunlap et al., 1973; Gerall et al., 1973). Furthermore, neonatal treatment with tamoxifen, a well-known estradiol receptor antagonist, was also found to reduce lordosis

behavior in female rats (Dohler et al., 1984), but the latter study was criticized because of the potential defeminizing effects of tamoxifen (Mathews et al., 1988). By using female mice carrying a targeted mutation of the aromatase gene (aromatase knockout or ArKO), we provided more robust evidence for a role of estradiol in the development of lordosis behavior (Bakker et al., 2002). Female ArKO mice showed significantly lower levels of lordosis behavior than wild type (WT) controls following adult ovariectomy and treatment with ovarian hormones. However, administration of estradiol during a specific prepubertal period (i.e., postnatal days P15-25) significantly enhanced lordosis behavior in response to male mounts, almost up to levels observed in WT females (Brock et al., 2011). Interestingly, administration of estradiol during the neonatal period (P5-P15) completely defeminized lordosis behavior in WT females thereby emphasizing that the same hormone can have strongly opposing effects depending on when during development it is present or administered (Brock et al., 2011). It also suggests that there are two different developmental time windows for a male- versus a female-typical sexual differentiation of the mouse brain with male sexual differentiation proceeding pre- and early postnatally and female sexual differentiation predominantly postnatally, starting in the second week of postnatal life, which actually coincides with the onset of ovarian hormone secretion by the ovaries (Mannan and O'Shaughnessy, 1991).

At present, the mechanisms, by which estradiol induces either a defeminization or feminization of the brain, are still unknown. There is however robust evidence that estradiol up-regulates the progesterone receptor (PR) through its receptor ERα (reviewed in Wagner, 2006). Interestingly, estradiol induces both the male- and female pattern in PR expression, i.e. higher PR expression in the male hypothalamus prenatally and early postnatally (E18-P10) in the anteroventral periventricular area (AVPV), the medial preoptic nucleus (MPO), and in the

ventromedial hypothalamus (P0-P20), whereas the female pattern in PR expression developed gradually throughout postnatal development, between P10 and P25 (Quadros and Wagner, 2008; Brock et al., 2010). These sex differences in PR expression over specific developmental periods might thus suggest a sex-specific role for PR and its substrate progesterone in male versus female brain sexual differentiation. At present, however, no conclusive evidence for a role of PR in brain sexual differentiation has been obtained. In addition, little is known about endogenous progesterone concentrations in the rodent brain during early development. Progesterone can either be locally produced or come from the circulation as it is secreted by the adrenal glands in both sexes (reviewed in Schumacher et al., 2014), and the ovaries starting around day 7 after birth in females (Mannan and O'Shaugnessy, 1991). Studies in male PRKO mice have provided opposite results, i.e. either an enhancement or an inhibition of male sexual behavior in adulthood (reviewed in Wagner, 2006). By contrast, in female PRKO mice, lordosis behavior was clearly disrupted but it could not be determined using the PRKO model whether it was due to a developmental defect since a functional PR is prerequisite for showing lordosis behavior (Lydon et al., 1995). Furthermore, studies in which either progesterone or its antagonist RU486 was administered to neonatal male rats, showed that male sexual behavior was disrupted by both treatments (reviewed in Wagner, 2006). Finally, in a more recent study (Forbes-Lorman et al., 2014), the hypothesis of a sex-specific role for PR was determined directly by comparing male and female rats following treatment with RU486 during the neonatal period (P1-P7). Male sexual behavior was significantly increased in males perhaps due to increased expression of androgen receptors and thus increased sensitivity to androgens, but no effects were observed in females regarding lordosis behavior. The latter finding might not be surprising in light of our observation that the critical period for female sexual differentiation is later in life, as well as that PR expression is still very low in the female hypothalamus between P1 and P7 (Brock et al., 2010).

Therefore, in the present study, we revisited the question of a potential sex-specific role for PR in male versus female brain sexual differentiation by taking advantage of a newly developed PR antagonist ZK137316, as well as that we treated male and female subjects during their respective critical periods, i.e. neonatally (P0-P10) for the males and prepubertally (P15-P25) for the females. RU486 has been criticized for its use as selective PR antagonist since it is known to interfere with glucocorticoid receptors, which is not the case for ZK137316 (Banaszak et al., 2000; Fuhrmann et al., 2000; Sanchez-Criado et al., 2000; Xu et al., 2000; Slayden et al., 2001). Because ZK137316 was never used before in mice, a pilot study was conducted first to determine its potential to inhibit lordosis behavior as well as the expression of hypothalamic NPY receptors (YR1) in adult female mice, both variables being strongly dependent on a functional PR (Lydon et al., 1995; Xu et al., 2000). Next, we treated groups of male and female mice with ZK during development and determined its effects on sexual behavior. Finally, we determined whether treatment with ZK during development had long-lasting effects on steroid receptor expression, PR and ERα expression in females, and PR and AR expression in males, since Forbes-Lorman et al. (2014) reported increased AR expression after neonatal RU486 treatment.

Methods

Animals

All experiments were conducted in accordance with the guidelines established by the institutional animal care and use committee of the National Institutes of Health "Guide for the Care and Use of Research Animals, Eight Edition", and were approved by the Ethical Committee for Animal Use of the University of Liege. The male and female *C57Bl/6j* mice used for the pilot experiment

were obtained from Charles River Breeding Laboratories (L'Arbresle, France) at the age of 8 weeks. The male and female *C57Bl/6j* mice used for the developmental experiment were obtained from a local breeding colony at the animal facility of the University of Liège. All experimental animals were housed under a reversed light/dark cycle (12h:12h light/dark; 21.00h lights on and 9.00 lights off) with food and water *ad libitum*

Ovariectomies and hormonal treatment: In adulthood, all experimental and stimulus females were bilaterally ovariectomized under general anaesthesia after an intraperitoneal injection (i.p.) of a mixture of ketamine (80mg/kg per mouse) and medetomidine (Domitor, Pfizer, 1 mg/kg per mouse). Mice received atipamezole (Antisedan, Pfizer, 4 mg/kg per mouse) subcutaneously (s.c.) at the end of the surgery in order to antagonize medetomidine-induced effects, thereby accelerating their recovery, as well as a s.c. injection of an analgesic (Temgesic, 0.05mg/kg per mouse). During ovariectomy, females received at the same time s.c. in the neck a 5-mm-long Silastic capsule (inner diameter: 1.57 mm; outer diameter: 2.41 mm; length: 5mm) containing crystalline 17β-estradiol (diluted 1:1 with cholesterol) to induce estrous levels of estradiol (for more details see Bakker et al., 2002). Females were allowed to recover for two weeks before the onset of the behavioral tests. All experimental and stimulus males were left gonadally intact.

Behavioral tests

Lordosis behavior: All lordosis tests were conducted in a Plexiglas aquarium (35cm long x 25cm high x 19cm wide) whose floor was covered with fresh sawdust. A sexually experienced male of the *C57Bl/6j* strain was placed alone in the aquarium and allowed to adapt for 15 min.

Subsequently, 3 hours after receiving a progesterone injection (500µg), the female was placed in the aquarium and the lordosis responses of the female to the mounts of the stimulus male were recorded. Only when the female arched her back and adopted a rigid posture standing on all four paws, it was scored as lordosis, meaning that lordosis was not scored based on whether or not the male was able to achieve an intromission. We have noticed before that some males can achieve intromission even though the female is not fully receptive (no arching of the back). The test lasted until the female received 10 mounts or 15 min had elapsed. Tests were performed once a week during the dark phase of the light cycle (between 4 and 6 h after lights out). At the end of each test, a lordosis quotient (LQ) was calculated by dividing the number of lordosis responses displayed by the female subjects by the number of mounts received (x100).

Male sexual behavior: All male sexual behavior tests were conducted in the same Plexiglas aquarium as described above. At the beginning of each test, the male was placed alone in the cage and allowed to adapt for 15 min. An estrous female (ovariectomized and brought into behavioral estrus) was then introduced into the cage and the latency to the first mount, intromission, and ejaculation, as well as the number of mounts, intromissions, and ejaculations, were recorded. The test lasted until ejaculation occurred or 30 min if no ejaculation was achieved. If a male never displayed a certain behavior within the 30 min test, the latency was scored as 1800 sec.

Mate preference: To assess mate preferences, we used a box (60 x 13 x 30 cm) that was divided into three compartments by placing opaque partitions. Each compartment was thus 20cm in length. The partitions contained perforated holes at a height of 8cm to facilitate the diffusion of odors from the two side compartments to the middle compartment. Tests were performed during the dark phase of the light cycle (6h after lights out). Animals were habituated to the three

compartment box only once on the day before the behavioral experiments by placing them in the middle compartment for 10 min (with no stimulus animals placed in the two side compartments). On the day of testing, stimulus animals were placed in the two side compartments with their own bedding to make the stimulus as odorous as possible. The subject was then introduced into the middle compartment containing no sawdust, and was observed for 9 min. The time spent poking its nose through the holes of the partition or actively sniffing the bottom of the partition was recorded with a stopwatch.

Non-reproductive behaviors: Because ZK137316 has not been used in mice before, we also determined whether it would affect other, non-reproductively-related behaviors. Therefore, we tested for general locomotor activity by using an open field as previously described (Dalla et al., 2004; Taziaux et al., 2007). The number of line crossings (from one rectangle to another) was recorded during 10 min. We considered the line crossed when all four legs had passed. Furthermore, we also determined the state of anxiety by using an elevated plus maze as previously described (Dalla et al., 2004). At the beginning of the test, each mouse was placed in the center area and subsequently the time spent in the open and closed arms was recorded for 10 min. In addition the number of entries into either the open or closed arms was registered. The mouse was considered to be in the open (or closed) arm when its four legs were no longer in the center area. These tests were conducted before mice were tested for their reproductive behavior.

Determine whether ZK blocks lordosis behavior and Y1R expression

ZK137316 (further referred to as "ZK") was kindly provided by Bayer Pharmaceuticals. Different generations of this antagonist have been previously used in various species, but not in

mice so far (Banaszak et al., 2000; Fuhrmann et al., 2000; Sanchez-Criado et al., 2000; Xu et al., 2000; Slayden et al., 2001) . We selected two doses of ZK (6 and 9 mg/kg) based on previous studies in rats (Xu et al., 2000; Sanchez-Craido et al., 2000). For reasons of comparison, we also tested RU486 (Mifespristone; M8046, Sigma-Aldrich, France) at the dose of 12mg/kg adapted from previous rat studies (Xu et al., 2000; Gaytan et al., 2003; Carillo-Martinez et al., 2011).

Adult *C57Bl/6j* female mice (8 weeks old) were ovariectomized and implanted with an estradiol capsule. Two weeks later, they were divided into 3 groups (n=7/8 per group). First, all females were tested for lordosis behavior during 3 consecutives tests (one test per week) to induce sufficient levels of lordosis 3h after receiving a s.c. injection with progesterone. Then, for the fourth test, females received either an injection of ZK at 6mg/kg (ZK6, n=8) or at 9mg/kg (ZK9, n=8) or RU486 at 12mg/kg (RU12, n=7) 2h before an injection with progesterone and thus 5h prior to the lordosis test. A fifth test was performed without ZK or RU486, but with progesterone, to determine whether lordosis behavior was restored to pre-treatment levels.

Finally, to determine whether ZK would affect Y1r mRNA levels in the hypothalamus**,** the same females as used in the behavioral tests, were injected with either ZK at 6mg/kg (ZK6, n=4) or 9 mg/kg (ZK9, n=4), RU486 at 12mg/kg (RU12, n=4) or OIL vehicle (OIL, n=8) 2h before receiving a progesterone injection and thus 5h before being euthanized by decapitation. This experiment was conducted one week after completing the behavioral tests. Brains were removed and the hypothalamus was dissected and then directly frozen in liquid nitrogen and stored at -80°C until RNA extraction and RT-PCR. Hypothalamic tissues were homogenized with TRIzol® Reagents (1ml/100mg tissue) (15596-026, Life technologies). After sitting 5 min at room temperature (RT), chloroform was added (0.2mL/mL of TRIzol® Reagent) and homogenates were incubated for 15 min. After centrifugation at 10.000g for 15 min at 4°C, the aqueous phase

was transfered to a fresh tube and incubated with isopropanol (0.5mL/mL of TRI Reagent) for 10 min at RT. Then, after centrigugation at 12.000g for 10 min at 4°C, the supernatant was removed and the RNA pellet washed by adding 0.5 mL of 70% ethanol. After vortex followed by centrifugation (7500 rpm for 5 min at 4°C), the RNA pellet was dried and resuspended in DEPC-treated water for RNA quantification. RNA concentration and quality were determined using a Nanodrop ND-1000 spectrophotometer (Nyxor biotech, Paris). Two microgrammes of total RNA were saved for further use in the reverse transcription reaction, performed in a total volume of 10 µl containing M-MLV Reverse Transcriptase (Promega), RNase Inhibitor (Promega) and oligo dT (Promega) mixed according to manufacturer's instructions with each dNTP (Promega). Two microlitres of reverse transcriptase (in DEPC-treated water) was then used in a 25-µl PCR volume containing: 1X GoTaq® buffer (Promega), 0.2 mM of each dNTP (Invitrogen), 0.4µM of each primer: for Y1r (forward: 5'-CTGATGGACCACTGGGTCTT-3'; reverse: 5'-CAGGAACGTCACCGAAGAAG-3'; Eurogentec) and for RPS11 (forward: 5'-ACATGTCTGTGCACCTGTCC -3'; reverse: 5'-AGATTCCCCTGAGACCGTCT -3'; Eurogentec), and 1.25 U of GoTaq® polymerase (Promega). PCR products were visualised after standard electrophoresis in a 2.5% agarose gel. Semi-quantification of the intensity of RT-PCR signals was carried out by a densitometric analysis on image from the electrophoresis gel with a computer-based image analysis system using the protocol for gel analysis (http://imagejdocu.tudor.lu/doku.php?id=video:analysis:gel_quantification_analysis) of NIH IMAGE, version 1.49 (Wayne Rasband, NIH, Bethesda, MD, USA).

Determine whether blocking PR during development affects behavior

Pregnant females were checked daily for parturition towards the end of pregnancy. The day of birth was designated as postnatal day (P) 0. Based on the results of the pilot experiment, we selected the dose of 6mg/kg ZK for the developmental study. Males were treated between P0 and P10 and females between P15 and P25. For each developmental period, we conducted 3 injections of ZK at 6mg/kg or OIL vehicle every 5 days. Thus, for the neonatal period, we injected s.c. ZK or OIL vehicle at P0, P5 and P10 and for the prepubertal period, we injected s.c. ZK or OIL vehicle at P15, P20 and P25. Treatments and developmental periods were not mixed between litters, thus one litter was either injected with ZK or oil neonatally or prepubertally. The total number of males injected were: OIL (n=23 from 5 litters) and ZK (n=23 from 6 litters) and the total number of females injected were: OIL (n=15 from 4 litters) and ZK (n=17 from 4 litters). Females used in the behavioral tests were ovariectomized in adulthood (P80) whereas males were left gonadally intact.

Tissue processing for steroid receptor immunohistochemistry

Perfusion Upon completion of all behavioral tests, male and female mice were anesthetized and perfused transcardially with saline followed immediately by 4% cold paraformaldehyde. Brains were removed and post-fixed in 4% paraformaldehyde for 2 hours. Brains were then cryoprotected in 30% sucrose/TBS solution during 72 h, frozen on dry ice and stored at -80°C until used. Brain sections were cut at 30µm thickness on a Leica CM3050S cryostat. Forebrains were cut coronally from the rostral telencephalon to the posterior hypothalamus. Sections were saved in four different series, placed in antifreeze solution, and stored at -20°C for later immunostaining.

Immunohistochemistry All incubations were carried out at room temperature and all washes were performed using Tris-buffered saline (TBS 0.05M) or Tris-buffered saline containing 0.1% Triton X-100 (TBST). Briefly, sections were rinsed and endogenous peroxidase activity was quenched by incubating the sections for 30 min with 3% hydrogen peroxide. Aspecific binding sites were then blocked by incubating sections for 30 min with 5% normal goat serum (NGS) (Dako Cytomation, Denmark). Sections were then incubated in TBST-5% NGS with either a rabbit polyclonal antibody against the progesterone receptor (1/2000, Dako; (Wagner et al., 1998), a rabbit polyclonal antibody against ERα (1/20000, C1355, EDM Millipore; Moffatt et al., 1998), both incubations overnight at 4°C, or a rabbit polyclonal antibody against the last 20 amino acid N-terminal sequence of the human AR (1/250; sc-816 (N-20), Santa Cruz Biotechnology Inc., CA, USA; Forbes-Lorman et al, 2014) for 48h at 4°C. Then, sections were incubated for 1h in a goat anti-rabbit biotinylated antibody (1/1000 in TBST; Dako Cytomation, Denmark) followed by a 1h incubation in avidin-biotin complex (1/800, ABC, Vector Laboratory) and finally reacted for 3 min with 3,3'diaminobenzidine tetrahydrochloride (0.04% DAB and 0.012% H_2O_2). Sections were washed, dried overnight, left in xylene (Sigma) for 10min and coverslipped using Eukit (Fluka, Steinheim, Germany).

Data analysis The total amount of immunoreactivity (ir) for each receptor was analyzed as previously described (Brock et al., 2010; 2015) Briefly, one representative section was selected for several brain nuclei and was digitized using a video camera (CFW 1612C; Scion Corp., Frederick, MD, USA) attached to a microscope (MTV-3, x 20 objective; Olympus, Tokyo, Japan). Then, immunoreactivity was quantified with a computer-based image analysis system using the particle-counting protocol of NIH IMAGE, version 1.49® (Wayne Rasband, NIH,

Bethesda, MD, USA). Digital images were made binary, and a manual threshold was used for discriminating the labeled material from the background. The total amount of PR-, ERα-, or AR-ir was measured in one entire field and then determined by measuring the area (µm²) covered by 'thresholded' pixels [i.e. those pixels with a grey level higher than a defined threshold density (specific immunoreactive staining)].

Statistical analyses: All statistical analyses were performed using Statistica® 10.0. First, it was determined whether the data followed a normal distribution. If so, then data were analyzed using oneway or repeated measures ANOVA. When appropriate, all ANOVAs were followed by Fisher's Least Significant difference (LSD) *post hoc* comparisons adapted for repeated-measures ANOVA. If not, then data were analyzed using Mann Whitney U tests. Regarding the pilot experiment on the ability of ZK to block lordosis behavior, we performed a t-test comparing the 4th test (i.e. injection of the PR antagonist) with the other tests (i.e. no PR antagonist). For the semi-quantification of Y1r mRNA level, we used the Kruskall-Wallis test. Regarding the developmental experiment, the lordosis data were analyzed using repeated measures ANOVA with treatment as independent variable and test as the repeated measure. The relative amount of PR-ir, ERα-ir and AR-ir was analyzed using oneway ANOVA for each brain region analyzed. Only significant ($P < 0.05$) effects detected by the ANOVAs and *post hoc* comparisons are presented here. Finally, we also estimated the effect size (d) of each result obtained (http://www.campbellcollaboration.org/resources/effect_size_input.php).

Results

Determine the ability of ZK to decrease lordosis behavior and Y1R mRNA expression

Lordosis behavior. As previously shown, lordosis behavior increased over repeated testing (Figure 1: tests 1-3). A single injection of ZK at 6mg/kg (Figure 1A), but not at 9mg/kg (Figure 1B) significantly reduced lordosis behavior. By contrast, a single injection of RU486 did not significantly reduce lordosis behavior (Figure 1C). Thus when comparing lordosis quotients of test 4 with those of test 3, the reduction was only significant when females were treated with 6 mg/kg of ZK ($t=3.16$, $p=0.015$, $d=4.0767$; figure 1A). Finally, lordosis behavior was completely restored in females previously treated with 6mg/kg of ZK when tested once more (test 5; $t=3.07$, $p=0.01$, $d=4.0300$; Figure 1A). An increase in lordosis behavior in the fifth test was also observed for females treated with 9mg/kg of ZK (Figure 1B) or with 12mg/kg RU486 (Figure 1C), but none of these reached statistical significance.

Y1R mRNA expression. An overall effect of treatment was observed in Y1R mRNA expression ($H(3,20)=11.65$, $p=0.008$). Subsequently, post-hoc analysis revealed that treatment with ZK at 6mg/kg significantly reduced Y1R messenger RNA levels in the hypothalamus of female mice ($p=0.009$, $d=9.01$) compared to OIL-treated female mice. Although Y1R mRNA levels seemed to be lower in females treated with ZK at 9mg/kg or RU486 at 12mg/kg compared to OIL, it was not statistically significant (ZK9: $p= 0.61$; RU486: $p=0.26$) (Figure 2).

Determine whether blocking PR during development affects sexual behavior and reproductive function

Males

Male sexual behavior Neonatal treatment with ZK had some minor effects on male sexual behavior, but only in the first mating test (Table 1). Thus, latencies to the first mount ($F_{(1,43)}$=8.79, p=0.0049, d=4.2303), intromission ($F_{(1,43)}$=4.22, p=0.046, d=2.9003) and ejaculation ($F_{(1,43)}$=5.48, p=0.024, d=3.2834) were significantly shorter in neonatally ZK-treated males compared to OIL-treated males. By contrast, the number of mounts with and without pelvic thrusts, intromissions, and ejaculations did not differ between neonatally ZK-treated and OIL-treated males in either of the 2 mating tests (Table 1).

Mate preference ANOVA indicated a significant effect of neonatal ZK treatment ($F_{(1, 84)}$ = 10.20 p=0.002, d=3.1216) as well as odor stimulus ($F_{(1, 84)}$ = 25.99 p<0.0001, d=3.1216), but no significant interaction ($F_{(1, 84)}$ = 0.1448 p= 0.7045), indicating that both ZK and OIL-treated males spent more time sniffing the side containing the estrous female over the side containing the male (Figure 3). Furthermore, neonatally ZK-treated males spent overall more time sniffing both sides of the three compartment box compared to OIL-treated males.

Non-reproductive behaviors Neonatal treatment with ZK had no effect on general locomotion in an open field nor did it affect the time spent in the open versus the closed arms in the elevated plus maze (data not shown).

PR and AR expression Neonatal treatment with ZK had no effect on PR expression in any of the brain areas analyzed. By contrast, a decrease was observed in AR-ir in the bed nucleus of the stria

terminalis (BST) (U=3.0, p=0.015, d=4.631) and a trend for a decrease in the medial preoptic area (mPOA) ($F_{(1,10)}$=4.34, p=0.06, d=2.9458) in males treated neonatally with ZK compared to OIL-treated males (Tables 2 and 3).

Females

Lordosis behavior Prepubertal treatment with ZK significantly reduced lordosis behavior when tested in adulthood after priming with ovarian hormones (Figure 4A). Statistical analysis with repeated measures ANOVA indicated a significant effect of treatment ($F_{(1,26)}$=8.17, p=0.008, d=0.24). Post hoc comparisons showed that lordosis behavior was particularly decreased in ZK-treated female mice in tests 2 (p=0.049, d=2.2197), 5 (p=0.02, d=3.7726) and 6 (p=0.02, d=3.7764). For the 3rd and 4th test, post hoc comparisons detected only a trend (test 3: p= 0.07, d=2.2565; test 4: p=0.06, d=2.8939).

Mate preference In contrast with lordosis behavior, mate preferences did not seem to be affected by prepubertal treatment with ZK. The ANOVA showed neither a significant effect of prepubertal treatment nor odor stimulus, indicating that neither OIL- nor ZK-treated females showed a clear mate preference (figure 4B).

Non-reproductive behaviors Prepubertal treatment with ZK had no effect on general locomotiony (Figure 5 A and B). By contrast, female mice treated prepubertally with ZK spent significantly

more time in the closed arm (U=61.5, p=0.021, d=3.3411) and less time in the open arm (U=61.5, p=0.021, d=3.3411) of the elevated plus maze than oil-treated females (Figure 5 C).

PR and ERα expression Blocking PR during prepubertal development had no effect on the relative amount of PR-ir or ERα-ir in any of the brain regions analyzed (Tables 2 and 4).

Discussion

In the present study we took advantage of a newly developed PR antagonist ZK137316 which does not to interfere with glucocorticoid receptors, to revisit the question of whether PR plays a sex-specific role in brain sexual differentiation. We found little evidence for such a role for PR in male sexual differentiation since no strong, long-lasting, effects on male sexual behavior were observed in males treated neonatally with ZK. By contrast, females treated with ZK during the prepubertal period showed significantly less lordosis behavior than OIL-treated controls. These results suggest a potential role for PR and consequently progesterone in brain feminization, at least for the development of lordosis behavior.

PR and lordosis behavior. Prepubertal treatment with the PR antagonist ZK137316 significantly decreased lordosis behavior in adult female mice primed with ovarian hormones. This decrease in lordosis behavior was not due to any long-lasting effects of ZK on the expression of PR or ERα, both receptors known to be crucial for the expression of lordosis behavior in adulthood (Laudau et al., 1978; Gonzalez-Mariscal et al., 1989; Lydon et al., 1996; Kudwa and Rissman, 2003), but

suggests some organizational role for PR and by consequence progesterone in lordosis behavior. Interestingly we observed reduced brain progesterone levels in female ArKO mice at P15 (unpublished data). If indeed progesterone is required for the development of lordosis behavior, a next logical step would be to inject progesterone in ArKO mice during the prepubertal period to determine whether it will restore lordosis behavior in these mice.

However, some caution is warranted in interpreting the results of the present study. In contrast with earlier studies on lordosis behavior (Bakker et al., 2002; 2006; Brock et al., 2011), we did not observe the typical increase in lordosis behavior in the OIL-treated females during repeated testing. Actually, quite surprisingly, lordosis quotients were quite similar between OIL- and ZK-treated females in the first test, i.e. around 30%. Then in the following tests, lordosis increased slightly in the OIL-treated females (but not significantly) whereas it decreased in prepubertally ZK-treated females. An alternative interpretation could thus be that testing for lordosis behavior, i.e. interacting with a male, might have induced some aversive effects in ZK-treated females. However, females were tested for mate preferences after the final lordosis test, and ZK-treated females showed like OIL-treated females a male-directed preference suggesting that sexual motivation was not affected by prepubertal ZK treatment. Finally, another explanation could be that peripubertal ZK treatment has affected cognitive abilities. Rat studies have shown that PR is expressed transiently in the developing cortex and progesterone might thus play a role in cortical development (Quadros et al., 2007). Although a recent study (Willing and Wagner, 2014) using PRKO mice suggested some role for PR in maturation of cortical connectivity and sensorimotor integration, clearly more research is needed to elucidate the role of PR in cortical development. Taken together, the most plausible explanation for the decrease in lordosis behavior following

prepubertal treatment with ZK remains that PR and thus progesterone are needed for the development of lordosis behavior.

PR and male sexual behavior The observation that PR expression is much higher in the male compared to the female hypothalamus during pre- and early postnatal development suggest a specific role for PR in the development of the male brain (Quadros and Wagner, 2008; Brock et al., 2010). Several studies have addressed this question by either using PRKO mice or injecting the well-known PR antagonist RU486. None of these studies have actually provided consistent results since either a decrease, no effect, or an increase in adult male sexual behavior was observed (reviewed in Wagner, 2006). One possible explanation could be that PR actually affects AR expression and perhaps thus AR-sensitive behaviors such as male copulatory behavior. For instance AR expression as well as male sexual behavior was increased in male PRKO mice (Schneider et al., 2005). In addition, in the recent study by Forbes-Lorman et al (2014), it was shown that blocking PR temporarily during neonatal development by injecting RU486 increased AR expression in several brain areas important for sexual behavior (i.e. BST, mPOA, dorsomedial VMH, medial amygdala) as well as male sexual behavior, the number of mounts and intromissions as well as shorter latencies to mount the female. In the present study, we observed shorter latencies to mount, intromit, and ejaculate in neonatally ZK-treated males compared to OIL-treated males, but no effects on the number of mounts, intromissions, or ejaculations. In addition, these effects were not long-lasting since there were no differences between the groups in the second mating test. The behavioral protocol used in our study is quite different from the one in the study by Forbes-Lorman and colleagues, in which males were gonadectomized, implanted with testosterone, tested three times for male sexual behavior and only the data of the

third test were provided, in addition to studying different species, mice versus rats. Furthermore, we found actually opposite effects regarding AR expression, i.e. a decrease in AR-ir in the BST and mPOA. Importantly we studied AR expression in gonadally intact male mice so it is possible that decreased AR expression actually reflects decreased testosterone levels in neonatally ZK-treated males. Since we found no deficits in male sexual behavior after neonatal ZK treatment, we did not pursue this study any further by castrating the males, treating them with testosterone and then re-test for male sexual behavior. Furthermore, neonatal ZK treatment had no effect on mate preference and thus sexual motivation, since they showed a clear preference for the estrous female over the sexually active male as did OIL-treated males. They spent more time sniffing both stimuli however, suggesting some hyperactivity in these males, but this was not confirmed in the open field test. Taken together, the question of why there is such a sex difference in PR expression during pre- and neonatal development remains yet to be answered. However, it should be kept in mind that even there is no strong evidence for PR or progesterone in male sexual differentiation, the male brain will be responsive to exogenous progesterone, such as progestins prescribed to women in late pregnancy to prevent premature delivery.

Increased anxiety in females. Locomotion measured in either an open field or elevated plus maze was not affected by prepubertal treatment with ZK. Nevertheless, prepubertally ZK-treated females spent more time in the closed than in the open arms of the EPM suggesting an increased state of anxiety. This result suggests that blocking progesterone receptors during the prepubertal period might have affected the neural circuits controlling stress responses in female mice. Indeed, numerous studies have shown a role of progesterone and/or its metabolites in stress and psychological diseases (for review see Wirth, 2011). Furthermore, levels of corticosterone as well

as sensitivity to stress have been reported to fluctuate over the estrous cycle, suggesting a specific role of ovarian hormones in stress, anxiety-like behavior and mood disorders (for review, see ter Horst et al., 2012). Finally, estradiol and progesterone might induce anxiety-like behavior since ovariectomized WT mice as well as ERKO/PRKO mice showed enhanced anxiety-like behavior (reviewed in Blaustein and Ismail, 2013). In the present study, PR and ERα expression was not affected by prepubertal ZK treatment thus the increase of anxiety-like behavior observed is most likely not due to any alteration in PR and ERα signaling. However, the possibility cannot be ruled out that other sex steroid receptors such as ERβ (Imwalle et al., 2005) or other neuronal targets important in anxiety were affected by prepubertal treatment with ZK. It remains however rather unlikely that the decrease in lordosis behavior was actually caused by increased anxiety since no specific stress responses were observed when ZK-treated females were tested for their lordosis behavior. There was no excessive running away from the male or any attempts to jump out of the testing apparatus. In addition, mate preferences were not affected: ZK-treated females showed like OIL-treated females a strong preference for the odors of a male.

In conclusion**,** the present study showed for the first time a potential role of PR in the development of female sexual behavior. However, more research is needed to further elucidate the role of PR and progesterone in the development of female sexual behavior.

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Legends to the Figures

Figure 1: Dose effect of ZK137316 on lordosis behavior in adult female mice. Following 3 pre-tests, 5h before the 4th test, adult female mice were injected s.c. with either (A) 6 mg/kg of ZK (ZK 6) or (B) 9 mg/kg (ZK 9) or (C)12 mg/kg of RU486 (RU 12). Then, a fifth test was performed with just estradiol and progesterone. Values are presented as means ± SEM. *$p<0.05$.

Figure 2: Dose effect of ZK137316 on Y1r mRNA hypothalamic levels in adult female mice. Adult females mice were injected either with OIL (n=8) or ZK at 6mg/kg (ZK 6) or ZK at 9mg/kg (ZK 9) or RU486 at 12mg/kg (RU 12) then killed 2h later and the hypothalamic level of NPY receptor (Y1r) and the ribosomal protein S11 (RPS11) mRNA were analyzed. (A)

Photomicrographs representing the Y1r and RPS11 mRNA electrophoresis gel. (B) Histogram showing the average of Y1r mRNA/ RPS11 mRNA ratio intensity for each group. Values are means ± SEM. * $p<0.05$

Figure 3: Mate preference of adult male mice treated with ZK137316 during the neonatal period. Time spent sniffing estrous female versus intact male volatile odors in the 3CB. Values are presented as means ± SEM. **$p<0.01$, ***$p<0.001$.

Figure 4: Sexual behavior of adult female mice treated with ZK137316 during the prepubertal period. (A) Lordosis quotients; (B) Time spent sniffing estrous female versus intact male volatile odors in the 3CB. Values are presented as means ± SEM. #$p<0.07$, *$p<0.05$

Figure 5: Locomotion and anxiety in adult female mice treated with ZK137316 during the prepubertal period. (A) Mean number of line crossings in the open field (OF). (B) Mean number of entries in the elevated-plus maze (EPM). (C) Mean number of time spent in either the open or closed arm. All data are expressed as means ± SEM. *$p<0.05$.

Table 1: Male sexual behavior after neonatal treatment with ZK or OIL. Mean number of mounts (MTS), intromissions (INTS) and ejaculations (EJAC) displayed during the 30-min test. Latencies are expressed in seconds; ML, Mount latency; IL, intromission latency; EL, ejaculation latency. **p<0.01, *p<0.05

		Numbers			***Latencies (s)***		
	Treatment	***MTS***	***INTS***	***EJAC***	***ML***	***IL***	***EL***
Test 1	***OIL (n=23)***	9.65 ± 2.54	107.78 ± 37.85	0.26 ± 0.09	787.17 ± 149.05	1392.26 ± 121.21	1717.30 ± 36.19
	ZK (n=22)	11.68 ± 1.89	114.77 ± 25.57	0.09 ± 0.10	304.5 ± 58.16 **	990.77 ± 157.42 *	1479.86 ± 96.62*
Test 2	***OIL (n=23)***	12.87 ± 1.96	196.26 ± 38.08	0.69 ± 0.09	432.87 ± 120.54	820 ± 157.32	1290.43± 100.75
	ZK (n=22)	15.04 ± 2.87	163.77 ± 33.94	0.64 ± 0.10	364.54 ± 131.16	674 ± 160.04	1146.6 ± 121.87

Table 2: Relative amount of PR-ir in different hypothalamic regions in female and male mice treated with ZK or OIL during the prepubertal (females) or neonatal (males) period. mPOA, medial preoptic area; AVPV, anteroventral periventricular area; ARC, arcuate nucleus; VMHvl, ventrolateral ventromedial hypothalamus.

	PR-ir in FEMALES treated PREPUBERTALLY		**PR-ir in MALES treated NEONATALLY**	
	OIL (n=5)	**ZK (n=5)**	**OIL (n=6)**	**ZK (n=6)**
mPOA	30337.6 ± 2215.6	36633.8 ± 2880.7	5428.17 ± 1371.65	3301.83 ± 1343.24
AVPV	16371.2 ± 2659	23539.8 ± 7267.3	3457 ± 565	4216.33 ± 2208.29
ARC	18029.6 ± 3899.2	14717.6 ± 2756.9	1700.2 ± 700.23	1222.33 ± 474.29
VMHvl	25514.4 ± 5595.5	21181 ± 9057.3	3203.33 ± 1266.71	2581.17 ± 1458.04

Table 3: Relative amount of AR-ir in different hypothalamic regions in male mice treated with ZK or OIL during neonatal development. mPOA,medial preoptic area; BST, bed nucleus of stria terminalis; LS, lateral septum; ARC, arcuate nucleus; DMH, dorsomedian hypothalamus, VMHvl, ventrolateral ventromedial hypothalamus; VMHdm, dorsomedial ventromedial hypothalamus; MeA, Medial Amygdala # $p<0.07$; * $p<0.05$: effect of treatment.

	AR-ir	
	OIL (n=6)	**ZK (n=6)**
mPOA	10127.67 ± 2784.48	3784.17 ± 1233.27 #
BST	328.5 ± 100.32	0 ± 0 *
LSV	170.33 ± 121.12	287 ± 138.48
ARC	4103.67 ± 1319.25	2674.83 ± 1616.27
DMH	165.83 ± 95.68	196.5 ± 156.20
VMHvl	2449.67 ± 1095.46	3856.17 ± 2889.67
VMHdm	491.67 ± 350.64	8.33 ± 8.33
MeA	1316.17 ± 875.70	2023.5 ± 952.22

Table 4: Relative amount of ERα-ir in different hypothalamic regions in female mice treated with ZK or OIL during prepubertal development. mPOA, medial preoptic area; AVPV, anteroventral periventricular area; ARC, arcuate nucleus; VMHvl, ventrolateral ventromedial hypothalamus. # $p<0.07$; * $p<0.05$: effect of treatment.

	ERα-ir	
	OIL (n=5)	**ZK (n=5)**
mPOA	22819.6 ± 7606.1	13026.2 ± 5315.1
AVPV	28531.8 ± 7625.7	20494.8 ± 4315.9
ARC	20071.2 ± 2429	22207 ± 4130
VMHvl	8504.4 ± 4670.4	9292 ± 6090.6

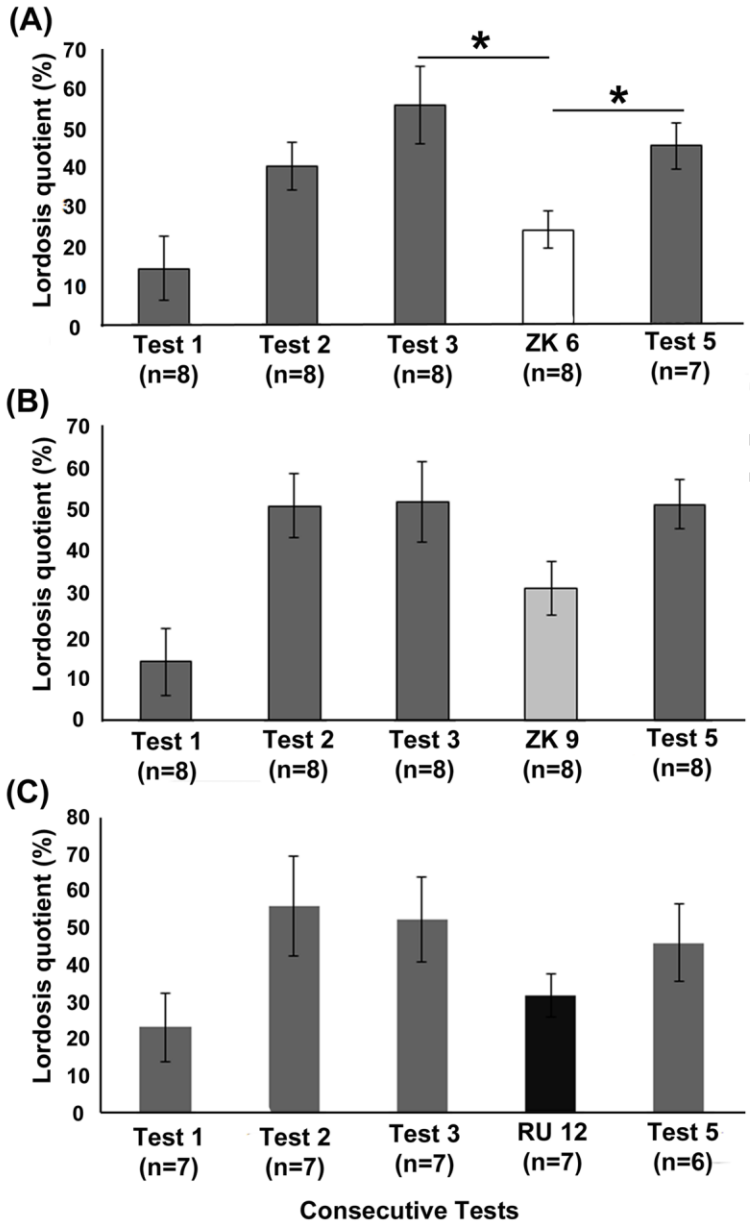

Fig. 1

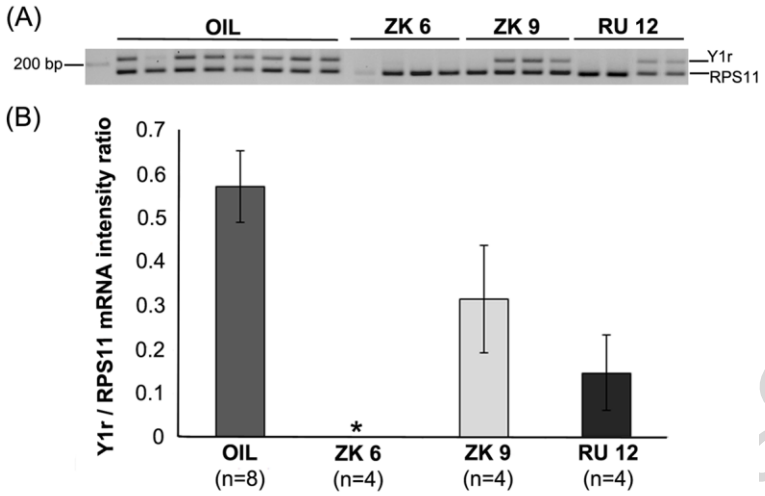

Fig. 2

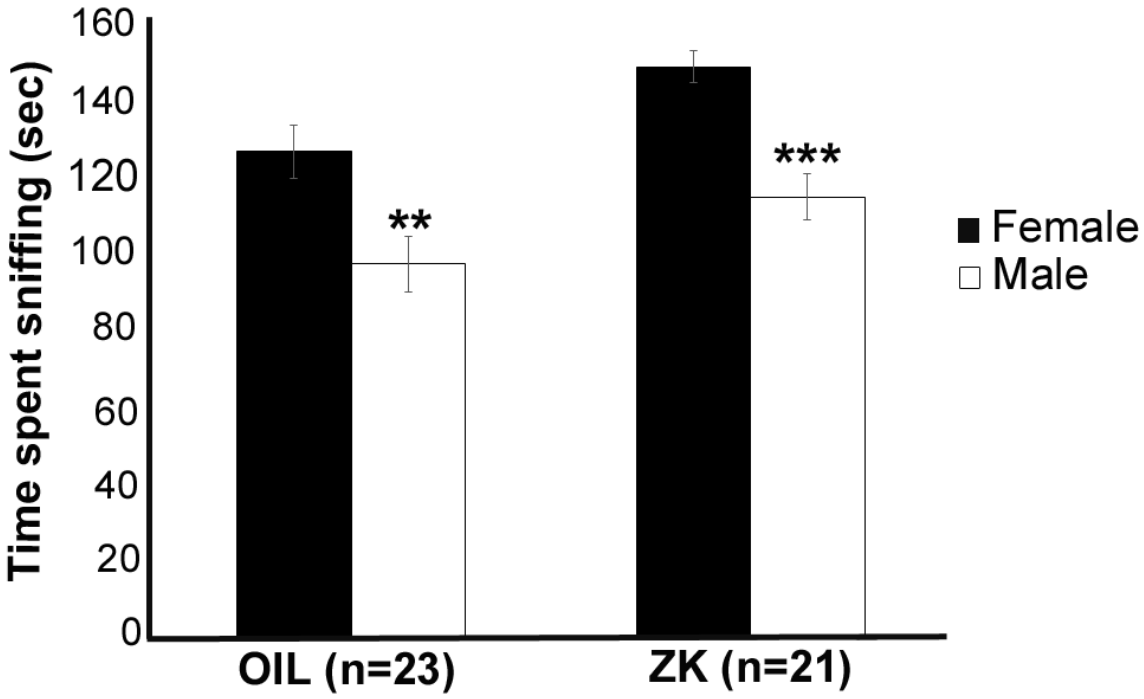

Fig. 3

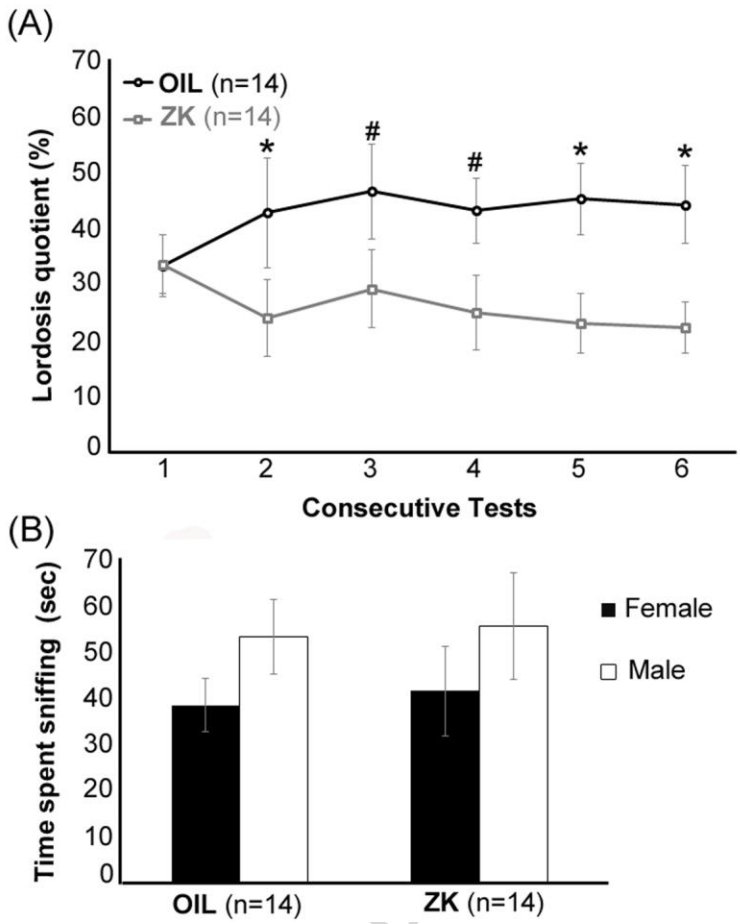

Fig. 4

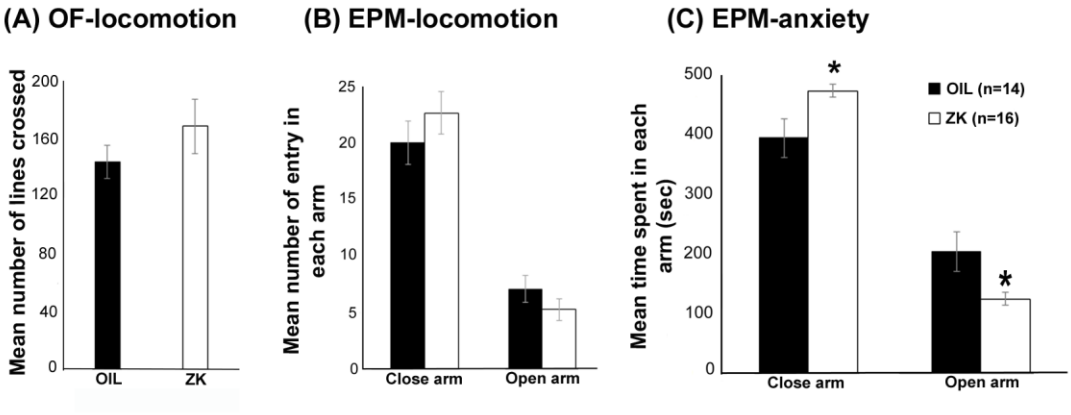

Fig. 5

Highlights

- Prepubertal treatment with the PR antagonist ZK137316 affected lordosis behavior in female mice
- Potential role for progesterone in the sexual differentiation of the female brain
- Neonatal treatment with the PR antagonist ZK137316 had only minor effects on male sexual behavior
- No clear role for progesterone or PR in male sexual differentiation