Revisiting the neural role of estrogen receptor beta in male sexual behavior by conditional mutagenesis

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Abstract

Estradiol derived from neural aromatization of gonadal testosterone plays a key role in the perinatal organization of the neural circuitry underlying male sexual behavior. The aim of this study was to investigate the contribution of neural estrogen receptor (ER) β in estradiol-induced effects without interfering with its peripheral functions. For this purpose, male mice lacking *ERβ* in the nervous system were generated. Analyses of males in two consecutive tests with a time interval of two weeks showed an effect of experience, but not of genotype, on the latencies to the first mount, intromission, pelvic thrusting and ejaculation. Similarly, there was an effect of experience, but not of genotype, on the number of thrusts and mating length. Neural *ERβ* deletion had no effect on the ability of males to adopt a lordosis posture in response to male mounts, after castration and priming with estradiol and progesterone. Indeed, only low percentages of both genotypes exhibited a low lordosis quotient. It also did not affect their olfactory preference. Quantification of tyrosine hydroxylase- and kisspeptin-immunoreactive neurons in the preoptic area showed unaffected sexual dimorphism of both populations in mutants. By contrast, the number of androgen receptor- and ERα-immunoreactive cells was significantly increased in the bed nucleus of stria terminalis of mutant males.

These data show that neural ERβ does not play a crucial role in the organization and activation of the neural circuitry underlying male sexual behavior. These discrepancies with the phenotype of global *ERβ* knockout models are discussed.

Keywords

Sex steroid hormones; Estrogen receptor beta; Nervous system; Estradiol; Sexual behavior; Conditional mutagenesis, Male reproduction; Sexual dimorphism

Introduction

In male rodents, sexual behavior is induced by olfactory cues. Pheromonal cues are transmitted from the main olfactory epithelium and vomeronasal organ to, respectively, the main and accessory olfactory bulbs, then to chemosensory responsive nuclei in the medial amygdala (MeA), bed nucleus of stria terminalis (BNST), and medial preoptic area (MPOA) where they are processed in behavioral responses. This neural circuitry is under the tight control of gonadal hormones. Estradiol derived from neural aromatization of perinatal testosterone induces irreversible masculinization and defeminization processes (Schwarz and McCarthy, 2011). Masculinization is the potentiation of neuroanatomical and behavioral patterns that are exhibited to a greater degree by males than females (e.g., preference for receptive females and copulatory behaviors). Defeminization is the loss of the ability to display female-typical behaviors such as preference for males and receptive mating posture (lordosis). The organizational effects of estradiol result in sex differences at the structural, neurochemical and molecular levels along the circuitry involved in the control of sexual behavior and reproductive functions. For instance, a cluster of calbindin-immunoreactive neurons in the MPOA, corresponding to the rat sexually dimorphic nucleus involved in sexual behavior, contains more cells in males than in females (Orikasa and Sakuma, 2010). Inversely, neurons expressing tyrosine hydroxylase (TH) or kisspeptin are less numerous in males compared to females in the anteroventral periventricular nucleus (AVPV), a subdivision of the medial preoptic area involved in the ovulatory surge of LH (Clarkson and Herbison, 2006; Kauffman et al., 2007; Simerly et al., 1985).

Estradiol acts mainly through two nuclear receptors (ER) α and β encoded by two different genes. Genetic studies highlighted the role of ERα in male reproduction and expression of male sexual behavior since global *ERα* knockout males are infertile and exhibit impaired

behavior (Ogawa et al., 1997; Ogawa et al., 1998; Wersinger et al., 1997). The involvement of ERβ in estradiol-induced effects needs further clarification. The analysis of the first genetic model with global *ERβ* deletion (Krege et al., 1998) showed that mutant males are fertile and display normal sexual behavior and olfactory preference (Kudwa et al., 2005; Ogawa et al., 1999). A transient effect of *ERβ* deletion was observed around the time of puberty since peripubertal mutants displayed delayed ejaculation behaviour (Temple et al., 2003). When mutant males were castrated at adulthood and primed with estradiol and progesterone, they displayed a higher lordosis behavior than wild-types (Kudwa et al., 2005). At the neuroanatomical level, it was found that the number of TH-immunoreactive cells was increased in the AVPV region of mutant males by comparison to wild-types (Bodo et al., 2006). This suggested that ERβ mediates the estradiol-induced defeminization of the male brain. Global *ERβ* deletion also affected the sexually dimorphic expression of ERα in the preoptic area (Temple et al., 2001). By contrast, in the BNST, the volume and neuronal number, which are more important in males than females, were not affected (Tsukahara et al., 2011). More recently, a global *ERβ* knockout mouse line, devoid of any *ERβ* transcript, was generated by using the Cre-loxP system (Antal et al., 2008). These mutant males are infertile and exhibit mildly impaired sexual behavior (Antal et al., 2012). They display higher numbers of mounts and intromissions as well as delayed ejaculation, but these deficits were improved by sexual experience. In this mouse model, the involvement of ERβ in the defeminization processes of the male brain has not been studied.

Although useful, the global genetic models limit the understanding of the neural contribution of ERβ, due to the ubiquitous nature of the gene deletion. Estrogens through ERβ which is expressed in the testis, epididymis and prostate (Saunders et al., 1998; van Pelt et al., 1999), play also a role in the physiology of the male urogenital tract (Imamov et al., 2004; Sar

and Welsch, 2000; Wahlgren et al., 2008). The present study was undertaken in order to investigate the neural implication of ERβ in the masculinization and defeminization of the neural circuitry underlying male sexual behavior, without interference with its peripheral functions. For this purpose, we generated a mouse line lacking *ERβ* in the nervous system by using Cre-loxP technology. Male sexual behavior was analyzed in both naïve and sexually experienced males in the presence of receptive females. The ability of males to adopt lordosis posture in response to mounts of stud males was also analyzed. The effects of neural *ERβ* mutation on the organization of TH- and kisspeptin-immunoreactive neurons located in the sexually dimorphic rostral periventricular area of the third ventricle (RP3V) were investigated. Finally, the potential impact of neural *ERβ* deletion on the expression of androgen receptor (AR) and ERα expression was evaluated in brain areas underlying male sexual behavior.

Material and methods

Animals

The ERβ^{NesCre} mouse line was obtained, on a C57BL/6J genetic background, by crossing floxed *ERβ* females in which exon 3 of *ERβ* was flanked by loxP sites (Antal et al., 2008) with floxed *ERβ* males expressing the Cre recombinase under the control of the rat nestin (Nes) promoter and neural-specific enhancer (Raskin et al., 2009) as recently described (Naulé et al., 2015). Cre-mediated excision of floxed exon 3 of the *ERβ* gene allows the deletion of all *ERβ* transcripts (Antal et al., 2008). Mutant mice (ERβ$^{fl/fl}$ carrying the NesCre transgene; ERβ^{NesCre}) and their control littermates (ERβ$^{fl/fl}$) were group-housed under a controlled photoperiod (12:12-h light–dark cycle – lights on at 7 am), maintained at 22°C, with free access to food and water. All studies were performed on 2-4 months old animals, in accordance with the European guidelines for use of experimental animals (Decree 87-848, 86/609/ECC). Experiments were performed accordingly, to minimize animal number and discomfort and were approved by the local Department of Animal Protection and Health.

PCR and RT-PCR

Neural *ERβ* invalidation was confirmed by both PCR and RT-PCR. The lack of antibodies specific enough against ERβ receptor (Snyder et al., 2010) did not allow analyses at the protein level. For PCR, detection of the Cre recombinase and *ERβ* alleles in DNA extracts from adult and neonatal brains was performed as previously described (Antal et al., 2008; Raskin et al., 2009). For RT-PCR, total RNAs were extracted from the brain and epididymis using Trizol reagent (Invitrogen, Carlsbad, USA). RNA (2 µg) was reverse transcribed using the Superscript III first strand Synthesis System (In vitrogen). PCR reactions were performed using the resulting cDNA, Taq DNA pol (In vitrogen), dNTPs (10 nM each), forward (5'-CAGAGAGACCCTGAAGAGGA-3') and reverse (5'-CCTTGAATGCTTCTTTTAAA-3')

primers for ERβ (Antal et al., 2008) and for GAPDH (forward: TGCACCACCAACTGCTTAGC; reverse: GGCATGGACTGTGGTCATGAG) in a MyCycler Thermal Cycler (Bio Rad, Marne la Coquette, France). The amplified cDNA fragments were separated by electrophoresis through a 1.5% agarose gel and stained by ethidium bromide.

Urogenital tract, hormone levels and fertility

Intact animals were sacrificed to collect blood and to weigh seminal vesicles. Sera were extracted and circulating levels of testosterone were measured by RIA at the hormonal assay platform of the laboratory of behavioral and reproductive physiology (UMR 7247 INRA/CNRS/Université François Rabelais) using ^{3}H-T, as previously described (Picot et al., 2014). The mean intra-assay coefficient of variation was 7% and assay sensitivity was 125 pg/ml.

To evaluate fertility, three months-old males (4 per genotype) were mated for 4 months. Each male was individually housed with two age-matched females. The number of pups and the interval from mating to the first litter were recorded.

Behavioral analyses

Tests were conducted under red-light illumination 2 hours after lights-off and videotaped for analyses.

<u>Male-typical behaviors of intact males</u>

Male sexual behavior

Intact animals were individually housed 3 days before the first test. Each male was tested in its home cage for 10 h after the introduction of an estrus female. They were tested twice with

a time interval of two weeks. Male sexual behavior was analyzed by scoring the latency and the frequency of mounts, intromissions, thrusts and ejaculation as previously described (Raskin et al., 2009). Estrus C57BL/6J females used as stimuli were ovariectomized under general anesthesia (xylazine 10 mg/kg / ketamine 100 mg/kg), implanted with SILASTIC implants filled with 50 µg of estradiol-benzoate (Sigma-Aldrich, Saint Louis, United States) in 30 µl of sesame oil and subcutaneously treated with 1 mg of progesterone (Sigma-Aldrich) in 100 µl of sesame oil four to five hours before the tests, as previously reported (Raskin et al., 2009). Female receptivity was verified before the beginning of experiments as following. Each female was put in the presence of a sexually experienced male, which was not in contact with a female for at least 1 week. The female was considered receptive when she displayed a lordosis posture with the four paws grounded, the hind region lifted and the back arched in response to male mounts.

For each male, the latencies from female introduction to the first mount, intromission, thrusting and to ejaculation were measured. The total number of mounts, without and with intromissions, and the total number of thrusts were measured. Mating length was defined as the time from the first mount to ejaculation.

Olfactory preference

Sexually experienced males were placed into an enclosed Plexiglas Y-maze without any stimuli, for 5 min on two consecutive days, to allow them to adapt to the apparatus. Animals were tested for mate preference on the third day by placing an anesthetized receptive female and gonadally intact male in boxes with perforated partitions at the end of each distal arm as previously described (Keller et al., 2006). The time spent sniffing at each partition was scored over the five-minute test. Results are expressed as a percentage of total time spent sniffing

male or female cues. The maze was cleaned with 10% ethanol between trials (Naulé et al., 2014).

Female-typical behaviors of castrated males primed with estradiol and progesterone

Lordosis behavior

Males (22 $ER\beta^{fl/fl}$ and 21 $ER\beta^{NesCre}$ mice) were castrated under general anesthesia (xylazine / ketamine). Four weeks later, they were tested for female sexual behavior in three consecutive tests conducted at one-week interval as previously described (Picot et al., 2014). Briefly, subjects were subcutaneously injected with estradiol-benzoate (10 µg dissolved in 100 µl of sesame oil) 48 h prior to the test and progesterone (1 mg in 100 µl of sesame oil) four hours before the tests. Experimental males were put in the presence of sexually experienced C57BL/6J male mice serving as stimulus animals. Tests ended when the subject received 20 mounts or after 20 minutes of test. The lordosis posture in response to stud male mounting was determined as mentioned above. The lordosis quotient was calculated only for the males, which received 20 mounts, as the number of times the male adopts a lordosis-like posture in response to stimulus male mount. A group of females (n = 10) used as controls for the lordosis behavior test was ovariectomized, implanted with estradiol and primed with progesterone as described above. They were tested twice in the presence of stud males at one-week interval.

Olfactory preference

Tests were performed as described above for intact males. Males castrated and primed with estradiol and progesterone were tested 1 week after lordosis behavior tests.

Immunohistochemistry

Intact males were sacrificed and transcardially perfused with a solution of 4% paraformaldehyde (PFA) in phosphate buffer (PB). Brains were post-fixed overnight in 4% PFA-PB, cryoprotected in sucrose and stored until analyses. They were sliced into coronal sections of 30 µm using a cryotome (Leica CM 3000). Kisspeptin, AR- and ERα-immunostaining were carried as previously described (Naulé et al., 2014; Picot et al., 2014). For TH- immunostaining, the sections were blocked for 2 h with 2% normal donkey serum (Sigma-Aldrich) in PB saline (PBS) containing 0.1% Triton-X100 and 0.25% human albumin, then incubated with polyclonal anti-TH antibody (1:5000; Chemicon, Temecula, United States) overnight. Immunofluorescence was performed with a CY3 donkey anti-rabbit secondary antibody (1:500, Jackson Immunoresearch, Montlucon, France) for 2.5 h at room temperature. After several rinses in PBS, sections were rinsed in water, dried, mounted in Fluoromount-G (Southern Biotechnology, Birmingham, AL, USA) under a coverslip and stored at 4°C in the dark.

The numbers of kisspeptin-, TH-, AR and ERα-immunoreactive cells were counted in anatomically matched sections identified using the Mouse Brain Atlas of Paxinos and Franklin (2001) as previously described (Naulé et al., 2014; Picot et al., 2014). Kisspeptin-immunoreactive cells were analyzed within each of the three subdivisions of the rostral periventricular area of the third ventricle within an area of 0.24 mm^2 including the AVPV nucleus (plates 28-29) and the preoptic periventricular nucleus, divided into rostral (plate 30) and caudal regions (plates 31-32). TH-immunoreactive cells were counted in the AVPV within an area of 0.24 mm^2 (plates 27-31). AR and ERα-immunoreactive cells were analyzed in the MPOA within an area of 0.68 mm^2, in the BNST within an area of 0.70 mm^2 (plate 30), and in the MeA within an area of 0.56 mm^2 (plate 47).

Statistical analysis

Data were expressed as mean ± S.E.M. Student's t-tests were used to determine the effect of genotype on circulating levels of testosterone, weight of seminal vesicles and fertility. Effect sizes were further estimated by calculating the Cohen's d (d = M/SD, where M is the mean of differences and SD is the standard deviation of differences; d = 0.2 is considered as a small effect size, d = 0.5 as a medium effect size and d = 0.8 as a large effect size). Two-way ANOVA was used to analyze the main effects of genotype and experience on male sexual behavior and lordosis quotient or genotype and stimulus on olfactory preference. Tukey post-hoc tests were used to determine group differences. Effect sizes were further estimated by calculating the eta-squared η^2 ($\eta^2 = SS_{effect} / SS_{total}$, where SS_{effect} is the sums of squares for the effect of interest and SS_{total} is the total sums of squares for all effects, interactions and errors, $\eta^2 = 0.02$ is considered as a small effect size, $\eta^2 = 0.13$ as a medium effect size and $\eta^2 = 0.26$ as a large effect size). As variances were not homogeneous between groups, TH-, kisspeptin-ERα- and AR immnunoreactivity was analyzed with Mann-Whitney nonparametric test. P values of less than 0.05 were considered to be significant.

Results

General characterization of the ERβ^{NesCre} mouse line

The selective neural deletion of *ERβ* was confirmed by RT-PCR. A 177 bp-amplified fragment was present at comparable levels of expression in the epididymis of ERβ$^{fl/fl}$ and ERβ^{NesCre} males (t = -2.543, p = 0.064, d = -2.540). This signal was present in the brain of ERβ$^{fl/fl}$ males, and highly reduced in their ERβ^{NesCre} littermates (-98%, t = 7.716, p = 0.001, d = 7.660; Fig. 1A-B). The NesCre transgene used triggers gene deletion in neural precursor cells by embryonic day 10.5, before gonadal differentiation. To ensure that excision of neural *ERβ* exon 3 was indeed efficient during the perinatal organization of the male brain, we performed a PCR analysis for *ERβ* alleles and Cre recombinase on DNA extracted from neonatal and adult brains. A small amplicon of 250 bp indicating Cre-mediated excision of *ERβ* exon 3 was found in the brain of both neonatal and adult males expressing the Cre recombinase (Fig. 1C). By comparison, a 850 bp signal corresponding to the floxed allele was seen in the brain of control littermates lacking the NesCre transgene.

Student's t tests showed no significant effect of the ERβ^{NesCre} mutation on circulating levels of testosterone (t = 0.731, p = 0.470, d = 0.250 versus ERβ$^{fl/fl}$; Fig. 1D). This result was corroborated by the unchanged weight of the androgen-dependent seminal vesicles in ERβ^{NesCre} males (t = -1.312, p = 0.198, d = -0.650 versus controls; Fig. 1E). In fertility tests, ERβ^{NesCre} males produced a total number of 45 ± 5.0 pups versus 46 ± 3.0 for ERβ$^{fl/fl}$ mice (t = 0.083, p = 0.936, d = 0.070). The interval from mating to first litter was also similar between the two genotypes (22 ± 0.4 days in ERβ^{NesCre} versus 21 ± 1.0 days in ERβ$^{fl/fl}$; t = 0.600, p = 0.570, d = 0.490).

Behavioral effects of neural *ERβ* invalidation

Male sexual behavior and olfactory preference of intact males

Comparable percentages of naïve males (86% of $ER\beta^{fl/fl}$ and 81% of $ER\beta^{NesCre}$ genotypes) displayed a full range of sexual behavior and reached ejaculation. Analyses of the latencies to the first behaviors showed a significant effect of experience for the latencies to the first mount ($F_{(1.28)} = 11.240$, $p = 0.002$, $\eta^2 = 0.141$), pelvic thrusting ($F_{(1,28)} = 12.400$, $p = 0.001$, $\eta^2 = 0.165$), intromission ($F_{(1.28)} = 13.450$, $p = 0.002$, $\eta^2 = 0.156$) and latency to ejaculation ($F_{(1.28)} = 14.730$, $p < 0.001$, $\eta^2 = 0.186$) as illustrated in Fig. 2A. There was no significant effect of genotype on the latencies of these behaviors (mount: $F_{(1.28)} = 2.100$, $p = 0.160$, $\eta^2 = 0.035$; intromission: $F_{(1.28)} = 2.650$, $p = 0.115$, $\eta^2 = 0.042$; thrusting: $F_{(1.28)} = 2.890$, $p = 0.100$, $\eta^2 = 0.046$; ejaculation: $F_{(1.28)} = 2.960$, $p = 0.096$, $\eta^2 = 0.043$). The number of mounts without (M) or with intromission (MI), the total number of thrusts and mating length for both naïve and experienced males were also quantified (Table 1). Two-way ANOVA showed a significant effect of experience for the number of thrusts ($F_{(1,28)} = 6.920$, $p = 0.014$, $\eta^2 = 0.105$) and mating length ($F_{(1,28)} = 4.520$, $p = 0.042$, $\eta^2 = 0.070$) but not of genotype ($F_{(1,28)} = 0.610$, $p = 0.441$, $\eta^2 = 0.010$ and $F_{(1,28)} = 0.710$, $p = 0.408$, $\eta^2 = 0.012$, respectively). There was no significant effect of experience (M: $F_{(1,28)} = 0.250$, $p = 0.622$, $\eta^2 = 0.005$; MI: $F_{(1,28)} = 0.850$, $p = 0.360$, $\eta^2 = 0.012$) or genotype (M: $F_{(1,28)} = 0.070$, $p = 0.799$, $\eta^2 = 0.001$; MI: $F_{(1,28)} = 1.410$, $p = 0.240$, $\eta^2 = 0.027$) on the other components of mating.

The ability of males to discriminate between male and female pheromones in tests using gonad-intact male versus estrus female was tested. There was a significant effect of stimulus ($F_{(1,25)} = 7.020$, $p = 0.023$, $\eta^2 = 0.211$) but not of genotype ($F_{(1,25)} = 0.350$, $p = 0.565$, $\eta^2 = 0.011$; Fig. 2B). The total time devoted to chemoinvestigation was not significantly different between sexually experienced $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males (128.06 ± 10.35 sec versus 150.38 ± 12.93 sec, respectively; $t = 0.601$, $p = 0.560$, $d = 0.360$).

Lordosis posture and olfactory preference of castrated males primed with estradiol and progesterone

The ability of males to exhibit a typical female posture after castration and priming with estradiol and progesterone was measured. The percentage of males receiving 20 mounts from experienced intact males over the three tests averaged 50% in $ER\beta^{fl/fl}$ males and 57% in $ER\beta^{NesCre}$ mice. Only 14% of $ER\beta^{fl/fl}$ and 16% of $ER\beta^{NesCre}$ genotypes exhibited a lordosis posture. Statistical analysis of the lordosis quotient (LQ) of these males across the three tests showed a significant effect of time ($F_{(2,62)} = 4.560$, $p = 0.014$, $\eta^2 = 0.113$), but not of genotype ($F_{(1,62)} = 0.690$, $p = 0.410$, $\eta^2 = 0.009$; Fig. 2C). The mean LQ of $ER\beta^{fl/fl}$ males averaged 15% at Test 2 but then decreased to 1% at Test 3 while it was comprised between 2.5% and 5% in $ER\beta^{NesCre}$ mice. To make sure that these low LQ were not due to experimental limitations, we assessed a group of control females in similar conditions. Females exhibited a lordosis behavior with an LQ equivalent to 33.8 ± 7.6% since Test 1; it increased to reach 71.0 ± 8.2% in Test 2 (paired Student's t test, t = -3.510, $p = 0.006$, d = -4.966).

The males were then subjected to olfactory preference tests. There was no significant effect of stimulus ($F_{(1,33)} = 0.050$, $p = 0.825$, $\eta^2 = 0.002$) or genotype ($F_{(1,33)} = 1.210$, $p = 0.290$, $\eta^2 = 0.033$), indicating that males of the two genotypes displayed no olfactory preference (Fig. 2D). The total time spent investigating the two cues was similar between the two genotypes (117.97 ± 9.03 sec versus 106.99 ± 4.98 sec, respectively; t = 1.098, p = 0.290, d = 0.570).

Neuroanatomical organization of the medial preoptic area

The medial preoptic area, a key target of perinatal estradiol, contains known sexually dimorphic neuronal populations. Global deletion of *ERβ* was shown to increase the number of TH neurons in the AVPV, a subdivision of the medial preoptic area, suggesting that ERβ is involved in brain perinatal feminization of this region (Bodo et al., 2006). Thus, it was

assessed whether neural *ERβ* invalidation alters the neuronatomical organization of TH-immunoreactive cells. In accordance with previous studies (Simerly et al., 1985), the number of TH-immunoreactive cells was greater (2.4-fold) in females than in $ERβ^{fl/fl}$ males (p = 0.034; Fig. 3A and C). $ERβ^{NesCre}$ males showed a male pattern since no significant differences were seen with their $ERβ^{fl/fl}$ littermates (p = 0.885). The number of kisspeptin-immunoreactive cells, another sexually dimorphic population, was then quantified in the three subdivisions of the rostral periventricular area of the third ventricle (RP3V). Data show sex differences, with females exhibiting 24 to 225-fold higher number of kisspeptin-ir neurons in the AVPV (p = 0.026), rostral (p = 0.034) and caudal (p = 0.028) periventricular nuclei than $ERβ^{fl/fl}$ males (Fig. 3B and D). Again, no significant differences were observed between $ERβ^{fl/fl}$ and $ERβ^{NesCre}$ males (p = 0.317, p = 0.102 and p = 0.278 for the AVPV, rPeN and cPeN, respectively; Fig. 3D).

Quantification of the number of ERα- and AR-immunoreactive cells

ERα and AR signaling pathways play an important role in the expression of male sexual behavior. It was thus evaluated whether neural *ERβ* invalidation altered the number of ERα- and AR-immunoreactive cells in the neural circuitry underlying this behavior. The number of ERα -immunoreactive cells was unchanged in the MeA (p = 0.275) and MPOA (p = 0.513) of $ERβ^{NesCre}$ males by comparison to $ERβ^{fl/fl}$ males (Fig. 4A-B). It was, however, significantly increased by 37% in the BNST (p = 0.050 versus $ERβ^{fl/fl}$ genotype). Similarly, the number of AR-immunoreactive cells was unaltered in the MeA (p = 0.827) and MPOA (p = 0.513) and significantly increased in the BNST of $ERβ^{NesCre}$ mice (+38%, p = 0.050 versus $ERβ^{fl/fl}$ genotype; Fig. 5A-B).

Discussion

In order to determine the relative contribution of neural sex steroid receptors in reproductive behaviors, a mouse line lacking neural *ERβ* was characterized. This genetic model was generated by using the same strategy and NesCre transgene previously described for the mouse line lacking neural *AR* gene (Raskin et al., 2009).

In naïve ERβ^{NesCre} males, the latencies and frequencies to perform the various components of copulatory behavior were not statistically different from those observed in their control littermates. Sexual experience ameliorated mating in both ERβ$^{fl/fl}$ and ERβ^{NesCre} males by reducing the latencies to behaviors and mating length, and by increasing the number of thrusts. ERβ^{NesCre} males exhibited also normal preference towards female olfactory cues. These results are in agreement with the lack of ERβ involvement in the masculinization of sexual behavior and olfactory preference previously reported for the initial global *ERβ* knockout model (Kudwa et al., 2005; Ogawa et al., 1999). They contrast with the recent global *ERβ* invalidation (Antal et al., 2008), which resulted in increased number of mounts and intromissions and delayed ejaculation, although sexual experience progressively restored these behavioral differences (Antal et al., 2012). As these mice were obtained from the same floxed model and similar genetic background as the present conditional model, we suggest that the mild behavioral deficiency induced by this global mutation was probably due to peripheral effects of *ERβ* deletion. The present conditional mutation did not alter male fertility and circulating levels of testosterone while the global *ERβ* deletion generated by Chambon's laboratory resulted in an infertile phenotype of unknown origin (Antal et al., 2008). Whether or not these global *ERβ* knockout males exhibit altered regulation of the hypothalamus-pituitary-gonad axis, which may in turn interfere with reproductive behaviors, has not been reported. In males, ERβ is expressed in somatic and germ cells of the testis (van Pelt et al., 1999) and seems to be involved in testosterone production (Dumasia et al., 2015).

It has been shown that *ERβ* is important in the defeminization processes induced perinatally by estradiol in the neural circuitry underlying male sexual behavior (Kudwa et al., 2005). Mutant males from the initial global *ERβ* knockout model (Krege et al., 1998), exhibited a higher lordosis quotient than the wild-type males, after adult castration and priming with estradiol and progesterone. In our conditional model, only a small percentage of $ER\beta^{NesCre}$ males castrated and primed with estradiol and progesterone, showed a lordosis posture. Furthermore, $ER\beta^{NesCre}$ males exhibiting lordosis behavior displayed a low LQ (5%) in Test 3 while a mean value of 25% was reported for global *ERβ* knockout mice (Kudwa et al., 2005). Moreover, this low expression of lordosis behavior by $ER\beta^{NesCre}$ males was random across tests, indicating that there was no effect of sexual experience. In similar conditions, a group of control females exhibited a high LQ reaching 71% since Test 2. This demonstrates that the low behavior displayed by $ER\beta^{NesCre}$ males was not due to experimental limitations.

At the neuroanatomical level, the number of TH-ir neurons was not modified in the AVPV of $ER\beta^{NesCre}$ males. These results contrast with previous studies showing increased number of TH-ir neurons in the AVPV of global *ERβ* knockout males (Bodo et al., 2006). However, analysis of kisspeptin neurons, another sexually dimorphic population of the RP3V, confirmed the lack of $ER\beta^{NesCre}$ mutation effect on the sexual differentiation of the AVPV.

The discrepancy between the effects of global versus conditional *ERβ* mutation on the behavioral and neuroanatomical defeminization of the AVPV can not be attributed to differences in the genetic background as both models were studied on a C57BL6 background (Bodo et al., 2006; Kudwa et al., 2005). A possible explanation could be that global *ERβ* invalidation altered somehow other important pathways such as neural ERα or downstream regulated neurotransmitters or neuropeptides, which in turn interfered with sexual brain

differentiation. Increased number of TH-ir neurons has been, indeed, reported in the preoptic area of *ERα* knockout males (Simerly et al., 1997). Alternatively, cell types other than neuronal and glial cells targeted by $ER\beta^{NesCre}$ could be responsible of the phenotype observed in global *ERβ* knockout mice. Microglia cells were recently shown to be important for estradiol-induced sexual differentiation of the preoptic area and copulatory behavior (Lenz et al., 2013).

Neural invalidation of *ERβ* resulted in increased number of AR- and ERα-immunoreactive neurons specifically in the BNST. Previous studies reported that ERβ modulates ERα expression in hypothalamic cells (Malikov and Madeira, 2013). Whether ERβ modulates the expression of both AR and ERα in the BNST needs further investigation. Nevertheless, such cross-regulations between sex steroid receptor signaling pathways are not uncommon since a similar increase of ERα-immunoreactive cell number was noticed in the MeA and MPOA of males lacking neural *AR* (Picot et al., 2014). It is unlikely that the increased amount of AR and ERα proteins in the BNST compensates for the lack of ERβ in the expression of sexual behavior. Indeed, *ERβ* was deleted along the neural circuitry underlying sexual behavior and no changes in AR- or ERα-immunoreactivity were observed in the MeA or MPOA. The BNST is involved in other behaviors such as anxiety-like behavior (Daniel and Rainnie, 2015). Administration of a selective ERβ agonist to ovariectomized female rats has an anxiolytic effect (Lund et al., 2005; Weiser et al., 2009). This anxiolytic-like effect was observed in wild-type female mice but not in global *ERβ* knockouts (Oyola et al., 2012; Walf et al., 2008), which exhibit increased anxiety-like behavior (Krezel et al., 2001). In agreement with these observations, neural deletion of *ERβ* results in increased anxiety-state level during the follicular phase in female mice (Naulé et al., 2015). In male mice, the involvement of ERβ in estrogen-modulated anxiety state still needs to be documented. Minor effects of gene

invalidation were reported for global *ERβ* knockout males (Krezel et al., 2001), while chronic administration of androgen metabolites with actions at ERβ decreased the anxiety state level in rats (Osborne et al., 2009). Future studies will address the effects of neural *ERβ* deletion on anxiety-like behavior and aggression, another BNST-linked behavior altered in global *ERβ* knockout males (Nomura et al., 2002).

These data together with previous studies suggest that testosterone might regulate male sexual behavior mainly through ERα- and AR-signaling pathways. First, global *ERα* knockout males exhibit a severe sexual deficiency as evidenced by their lack of olfactory cues discrimination and partner preference (Wersinger and Rissman, 2000), increased latencies to mount, thrust and intromit and inability to ejaculate (Ogawa et al., 1997; Ogawa et al., 1998; Wersinger et al., 1997). It remains however to clarify whether the lack of ejaculation can be attributed solely to central effects of *ERα* mutation since this receptor plays also a role in the physiology of the male urogenital tract (Hess et al., 1997; Joseph et al., 2010). Second, neural invalidation of *AR* results also in sexual deficiency (Raskin et al., 2009). Unlike global *ERα* knockout males, males lacking the neural *AR* exhibit normal olfactory preference and are able to reach ejaculation in the C57BL6/J background, despite longer latencies to initiate the mounting and thrusting behaviors and reduced number of efficient mounts even after a first sexual experience (Picot et al., 2014). Neuroanatomical analyses of sexually dimorphic populations in brain areas underlying reproductive behaviors strongly suggest that the neural *AR* is not involved in their perinatal organization, but can rather mediate their activation during adulthood (Marie-Luce et al., 2013; Picot et al., 2014). In the spinal sites involved in erection and ejaculation, the neural AR plays a key role in postnatal differentiation and adult maintenance of the spinal nucleus of the bulbocavernosus and gastrin-releasing peptide neuron systems (Raskin et al., 2012; Sakamoto et al., 2014). These data are in good agreement

with the ontogeny of AR expression showing that this receptor is expressed after the perinatal period in both brain and spinal areas underlying male-typical behavior (Juntti et al., 2010; Smith et al., 2012).

Therefore, the ERα may play the main role in the perinatal organization of the brain circuitry underlying sexual behavior. The AR may act postnatally in the spinal cord and lately during pubertal/adult periods at both spinal and brain sites to activate the sexual circuitry. In this context, ERβ is not required for the organization and activation of sexual behavior. Previous studies suggested a role of this receptor in the timing of male sexual behavior at puberty (Temple et al., 2003). This together with our recent work, showing that neural *ERβ* deletion alters the timing of pubertal maturation in females (Naulé et al., 2015), suggest transient prepubertal functions for ERβ in both sexes. Further studies will characterize the pubertal phenotype of $ERβ^{NesCre}$ males.

In conclusion, the evaluation of neural effects of ERβ by using a conditional knockout model indicates that this receptor is not involved in the masculinization and defeminization of sexual behavior and related brain areas. ERα appears then as the dominant estrogen receptor mediating perinatal effects of estradiol, and AR and ERα might play complementary roles in the full expression of male sexual behavior. Since the $ERβ^{NesCre}$ mouse line displayed modifications in ERα and AR in the BNST, it will be therefore very useful for the investigation of the mechanisms underlying neural ERβ involvement in mood and aggressive behaviors without interference with male reproductive functions.

Acknowledgements

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Figure legends

Fig. 1. Characterization of the ERβ^{NesCre} mouse line. (**A**) RT-PCR of total RNAs obtained from the brain (Br) and epididymis (Ep). The representative gel shows the presence of the 177 base pair (bp) amplified fragment in the epididymis of both genotypes and only in the brain of control ERβ$^{fl/fl}$ males. DNA size markers at 50 bp increments are shown in the left column. (**B**) Quantitative data normalized to GAPDH from 3 males per genotype. $^{**}p < 0.01$ versus ERβ$^{fl/fl}$ brain. (**C**) PCR analyses performed on the brain of three neonates and four adults, obtained from the same litters, respectively. Up: PCR analysis showing the presence of the floxed ERβ allelle (850 bp) in the neonatal and adult brain of ERβ$^{fl/fl}$ mice (2, 3, 5, 7). The small amplicon of 250 base pair (bp) indicating Cre-mediated excision of exon 3 was present in the neonatal and adult brain of ERβ^{NesCre} littermates (1, 4, 6). Down: PCR analysis showing the presence of Cre recombinase in the neonatal and adult brain of ERβ^{NesCre} mice expressing the excised *ERβ* allele (1, 4, 6). DNA size markers at 100 bp increments are shown in the left column. (**D**) Circulating levels of testosterone in ERβ$^{fl/fl}$ and ERβ^{NesCre} males (n = 10 per genotype). (**E**) Weight of seminal vesicles (SV) expressed as percentage of body weight (bw) in male mice (n = 10 per genotype).

Fig. 2. Effects of neural *ERβ* invalidation on sexual behavior and olfactory preference in males. (A) Latencies to the first mount (Mo), thrust (Th), intromission (In), and ejaculation (Ej) of intact ERβ$^{fl/fl}$ and ERβ^{NesCre} males in Tests 1 and 2 (n = 13-17 animals per genotype). $^{a}p < 0.05$ versus Test 2. (**B**) Time spent chemoinvestigating gonad-intact male (M) versus estrus female (F) expressed as percentage of the total time chemoinvestigating (n = 9-10 males per genotype). $^{a}p < 0.05$ versus female stimulus. (**C**) Lordosis quotient of castrated males supplemented with estradiol and progesterone in three successive tests (n = 6-10 males per genotype). (**D**) Time spent chemoinvestigating intact males (M) versus estrus females (F),

expressed as percentage of total time spent chemoinvestigating, after castration and supplementation with estradiol and progesterone (n = 9-10 per genotype).

Fig. 3. Tyrosine hydroxylase (TH) and kisspeptin immunoreactivity in ERβ$^{fl/fl}$ and ERβ^{NesCre} males. (**A-B**) Representative immunostaining of TH (**A**) and kisspeptin (**B**) in ERβ$^{fl/fl}$ males, their mutant littermates (ERβ^{NesCre}) males and in control females. Scale bar = 100 μm. (**C-D**) Quantitative data for TH- in the anteroventral periventricular nucleus (AVPV) (**C**) and kisspeptin-immunoreactivity in the AVPV, rostral (rPeN) and caudal (cPeN) periventricular nuclei (**D**) are expressed as mean values ± S.E.M for 4 animals per group. $^{a}p < 0.05$ versus ERβ$^{fl/fl}$ males.

Fig. 4. Quantification of ERα-immunoreactive cell number in brain areas of ERβ$^{fl/fl}$ and ERβ^{NesCre} males. (**A**) Representative anti-ERα immunostaining in the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) of ERβ$^{fl/fl}$ and ERβ^{NesCre} males. Scale bar = 100 μm. AC, anterior commissure. (**B**) Quantitative data for the number of ERα-immunoreactive (ir) cells are expressed as mean values ± S.E.M for 3-4 animals per genotype. $^{a}p < 0.05$ versus ERβ$^{fl/fl}$ males in the BNST.

Fig. 5. Quantification of AR-immunoreactive cell number in brain areas of ERβ$^{fl/fl}$ and ERβ^{NesCre} males. (**A**) Representative anti-AR immunostaining in the medial amygdala (MeA), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) of ERβ$^{fl/fl}$ and ERβ^{NesCre} males. Scale bar = 100 μm. AC, anterior commissure. (**B**) Quantitative data for the number of AR-immunoreactive (ir) cells are expressed as mean values ± S.E.M for 3-4 animals per genotype. $^{a}p < 0.05$ versus ERβ$^{fl/fl}$ males in the BNST.

Table legends.

Table 1. Quantification of the sexual behavior displayed by naïve and sexually experienced ERβ$^{fl/fl}$ and ERβ^{NesCre} males. The number of mounts without (M) or with intromission (MI), the total number of thrusts (Th) and mating length are shown for males (n = 13-17 per genotype) tested in Tests 1 and Test 2. [a]$p < 0.05$ versus Test 1.

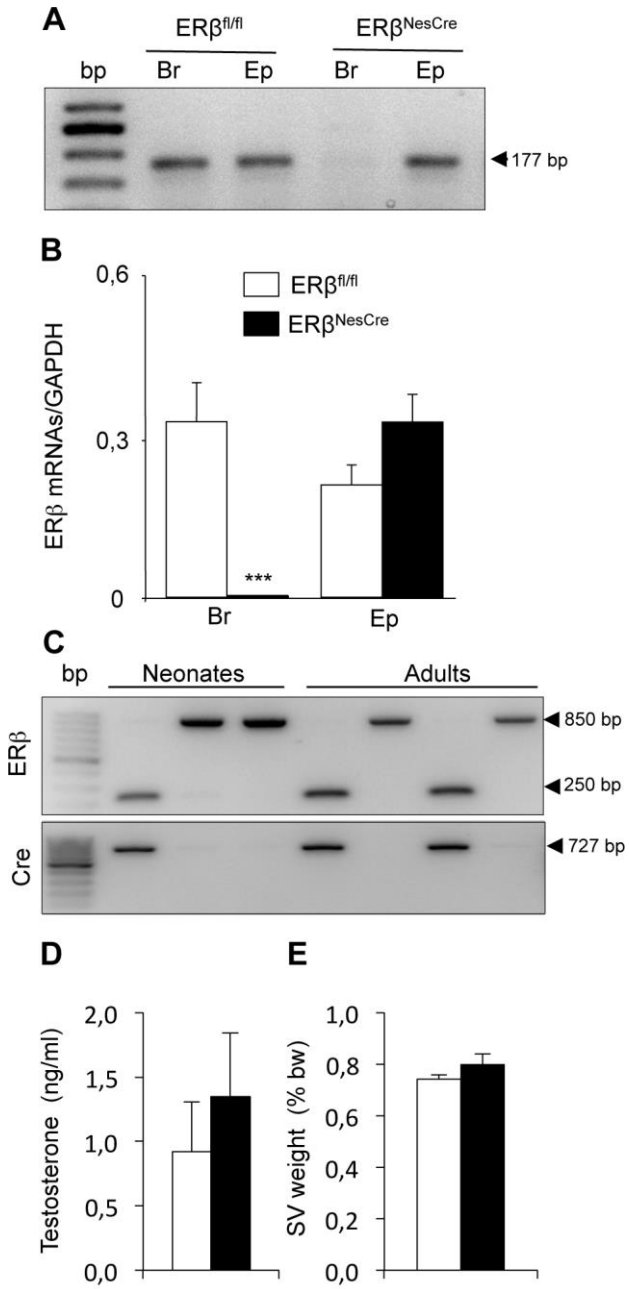
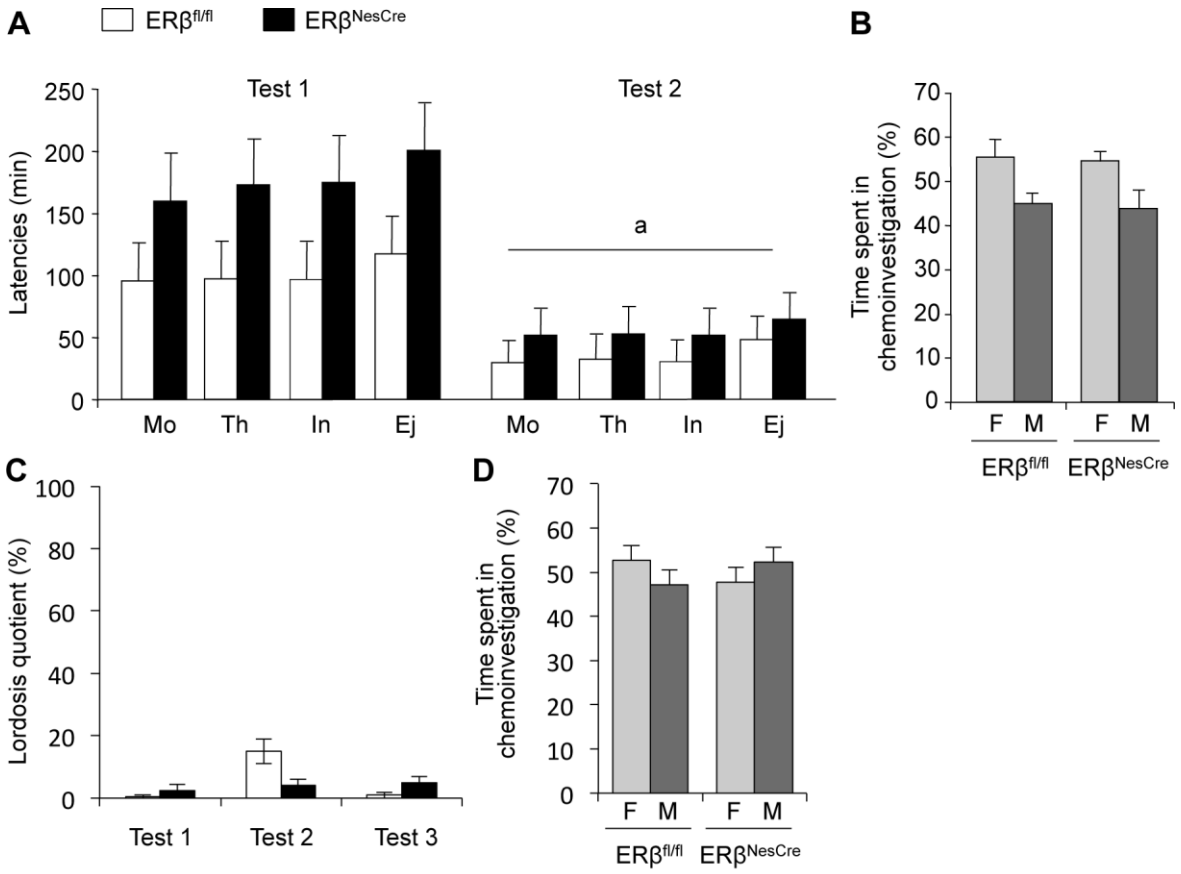

A
ERβfl/fl
ERβNesCre
bp
Br
Ep
Br
Ep
177 bp
B
ERβfl/fl
ERβNesCre
ERβ mRNAs/GAPDH
0,6
0,3
0
***
Br
Ep
C
bp
Neonates
Adults
ERβ
Cre
850 bp
250 bp
727 bp
D
Testosterone (ng/ml)
2,0
1,5
1,0
0,5
0,0
E
SV weight (% bw)
1,0
0,8
0,6
0,4
0,2
0,0

Figure 1



686 **Figure 2**

687

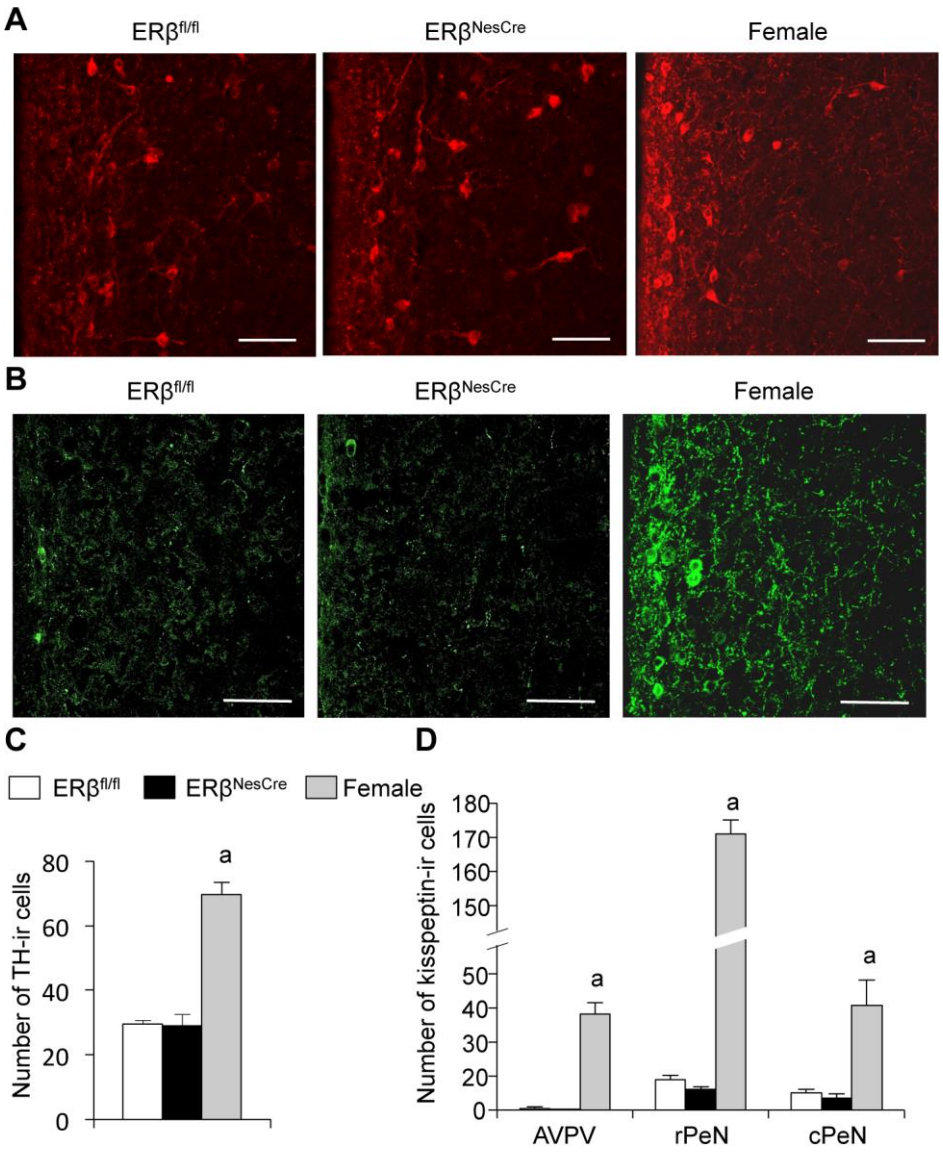

A
ERβfl/fl
ERβNesCre
Female
B
ERβfl/fl
ERβNesCre
Female
C
ERβfl/fl
ERβNesCre
Female
Number of TH-ir cells
0
20
40
60
80
a
D
Number of kisspeptin-ir cells
0
20
30
40
50
150
160
170
180
a
a
a
AVPV
rPeN
cPeN

Figure 3

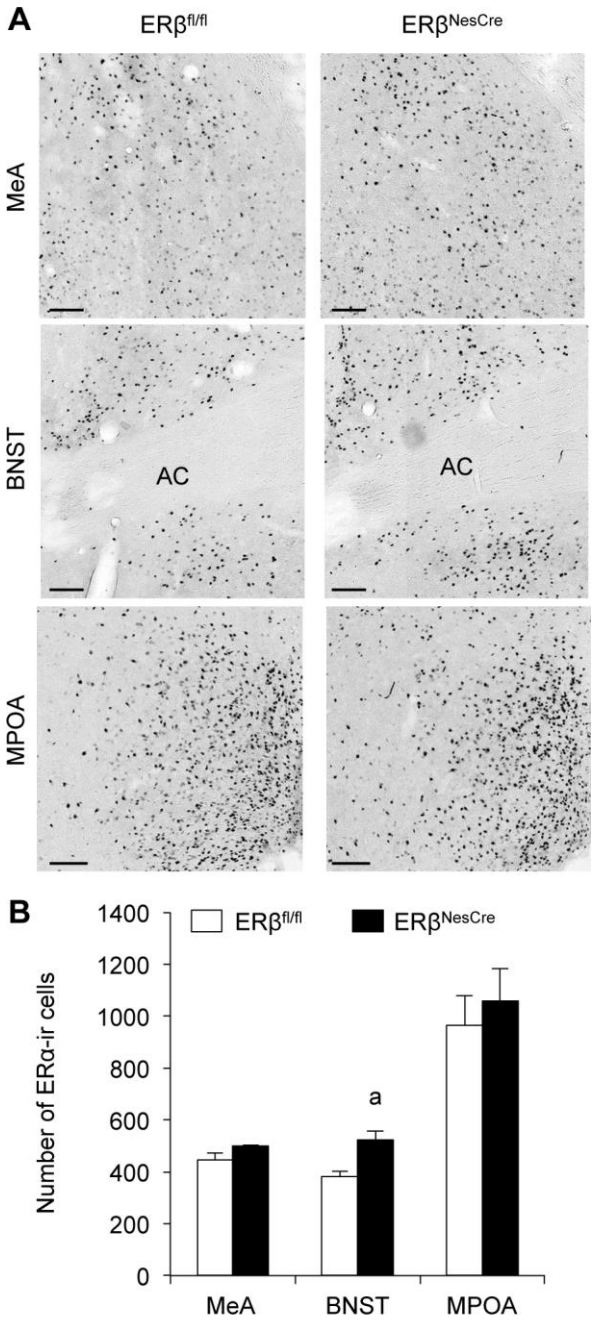


Figure 4

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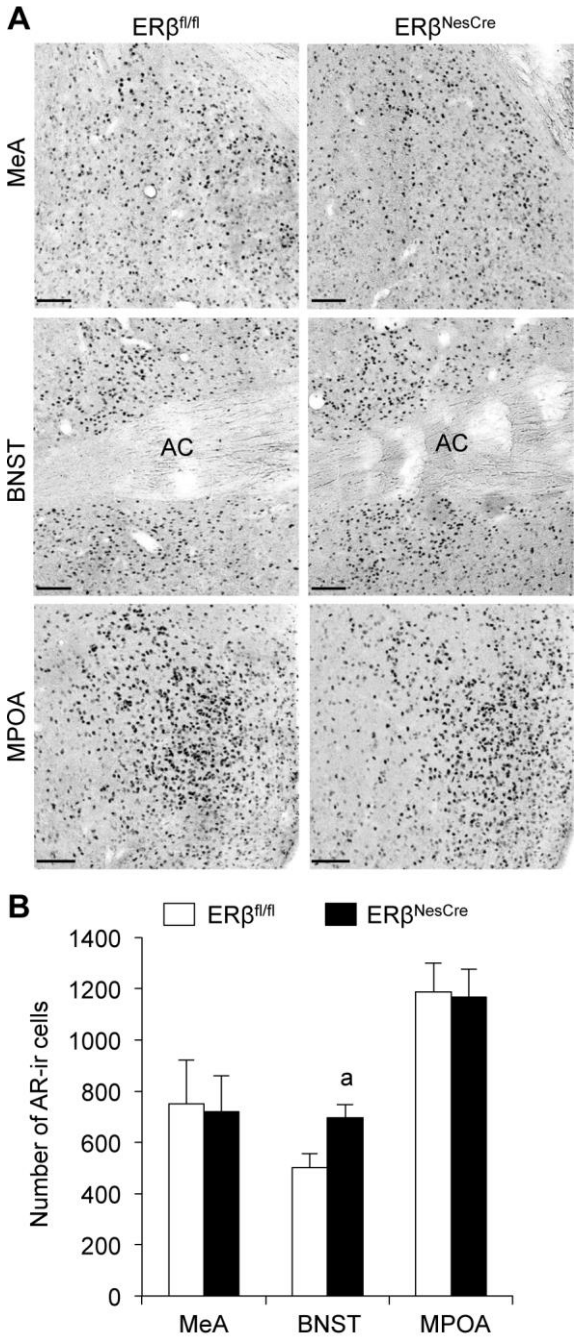
A
ERβfl/fl
ERβNesCre
MeA
BNST
MPOA
AC
AC
B
ERβfl/fl
ERβNesCre
Number of AR-ir cells
1400
1200
1000
800
600
400
200
0
a
MeA
BNST
MPOA

692

Figure 5