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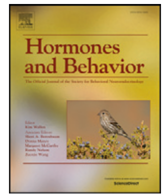

Revisiting the neural role of estrogen receptor beta in male sexual behavior by conditional mutagenesis

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A B S T R A C T

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.

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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, 2008). 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

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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, 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 behavior (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), also play 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 $ER\beta^{NesCre}$ 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 ($ER\beta^{fl/fl}$ carrying the NesCre transgene; $ER\beta^{NesCre}$) and their control littermates ($ER\beta^{fl/fl}$) 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 (Invitrogen). PCR reactions were performed using the resulting cDNA, Taq DNA pol (Invitrogen), 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: GGCATGGA CTGTGGTCATGAG) 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 month-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 h after lights-off and videotaped for analyses.

Male-typical behaviors of intact males

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/6 J 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 4 to 5 h before the tests, as previously reported (Raskin et al., 2009). Female receptivity was verified before the beginning of experiments as follows. 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

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