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

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1 **Title page**

2 **Title:** Revisiting the neural role of estrogen receptor beta in male sexual behavior by
3 conditional mutagenesis

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26 **Abstract**

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

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

46

47 **Keywords**

48 Sex steroid hormones; Estrogen receptor beta; Nervous system; Estradiol; Sexual behavior;

49 Conditional mutagenesis, Male reproduction; Sexual dimorphism

50

51 **Introduction**

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

72

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

76 behavior (Ogawa et al., 1997; Ogawa et al., 1998; Wersinger et al., 1997). The involvement of
77 ER β in estradiol-induced effects needs further clarification. The analysis of the first genetic
78 model with global *ER β* deletion (Krege et al., 1998) showed that mutant males are fertile and
79 display normal sexual behavior and olfactory preference (Kudwa et al., 2005; Ogawa et al.,
80 1999). A transient effect of *ER β* deletion was observed around the time of puberty since
81 peripubertal mutants displayed delayed ejaculation behaviour (Temple et al., 2003). When
82 mutant males were castrated at adulthood and primed with estradiol and progesterone, they
83 displayed a higher lordosis behavior than wild-types (Kudwa et al., 2005). At the
84 neuroanatomical level, it was found that the number of TH-immunoreactive cells was
85 increased in the AVPV region of mutant males by comparison to wild-types (Bodo et al.,
86 2006). This suggested that ER β mediates the estradiol-induced defeminization of the male
87 brain. Global *ER β* deletion also affected the sexually dimorphic expression of ER α in the
88 preoptic area (Temple et al., 2001). By contrast, in the BNST, the volume and neuronal
89 number, which are more important in males than females, were not affected (Tsukahara et al.,
90 2011). More recently, a global *ER β* knockout mouse line, devoid of any *ER β* transcript, was
91 generated by using the Cre-loxP system (Antal et al., 2008). These mutant males are infertile
92 and exhibit mildly impaired sexual behavior (Antal et al., 2012). They display higher numbers
93 of mounts and intromissions as well as delayed ejaculation, but these deficits were improved
94 by sexual experience. In this mouse model, the involvement of ER β in the defeminization
95 processes of the male brain has not been studied.

96
97 Although useful, the global genetic models limit the understanding of the neural
98 contribution of ER β , due to the ubiquitous nature of the gene deletion. Estrogens through ER β
99 which is expressed in the testis, epididymis and prostate (Saunders et al., 1998; van Pelt et al.,
100 1999), play also a role in the physiology of the male urogenital tract (Imamov et al., 2004; Sar

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

113

114 **Material and methods**

115 **Animals**

116 The $ER\beta^{NesCre}$ mouse line was obtained, on a C57BL/6J genetic background, by crossing
117 floxed $ER\beta$ females in which exon 3 of $ER\beta$ was flanked by loxP sites (Antal et al., 2008)
118 with floxed $ER\beta$ males expressing the Cre recombinase under the control of the rat nestin
119 (Nes) promoter and neural-specific enhancer (Raskin et al., 2009) as recently described
120 (Naulé et al., 2015). Cre-mediated excision of floxed exon 3 of the $ER\beta$ gene allows the
121 deletion of all $ER\beta$ transcripts (Antal et al., 2008). Mutant mice ($ER\beta^{fl/fl}$ carrying the NesCre
122 transgene; $ER\beta^{NesCre}$) and their control littermates ($ER\beta^{fl/fl}$) were group-housed under a
123 controlled photoperiod (12:12-h light–dark cycle – lights on at 7 am), maintained at 22°C,
124 with free access to food and water. All studies were performed on 2-4 months old animals, in
125 accordance with the European guidelines for use of experimental animals (Decree 87-848,
126 86/609/ECC). Experiments were performed accordingly, to minimize animal number and
127 discomfort and were approved by the local Department of Animal Protection and Health.

128

129 **PCR and RT-PCR**

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

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

144

145 **Urogenital tract, hormone levels and fertility**

146 Intact animals were sacrificed to collect blood and to weigh seminal vesicles. Sera were
147 extracted and circulating levels of testosterone were measured by RIA at the hormonal assay
148 platform of the laboratory of behavioral and reproductive physiology (UMR 7247
149 INRA/CNRS/Université François Rabelais) using $^3\text{H-T}$, as previously described (Picot et al.,
150 2014). The mean intra-assay coefficient of variation was 7% and assay sensitivity was 125
151 pg/ml.

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

155

156 **Behavioral analyses**

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

159

160 Male-typical behaviors of intact males

161 *Male sexual behavior*

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

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

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

180

181 *Olfactory preference*

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

188 male or female cues. The maze was cleaned with 10% ethanol between trials (Naulé et al.,
189 2014).

190

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

192 *Lordosis behavior*

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

207

208 *Olfactory preference*

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

211

212 **Immunohistochemistry**

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

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

236

237 **Statistical analysis**

238 Data were expressed as mean \pm S.E.M. Student's t-tests were used to determine the effect of
239 genotype on circulating levels of testosterone, weight of seminal vesicles and fertility. Effect
240 sizes were further estimated by calculating the Cohen's d ($d = M/SD$, where M is the mean of
241 differences and SD is the standard deviation of differences; $d = 0.2$ is considered as a small
242 effect size, $d = 0.5$ as a medium effect size and $d = 0.8$ as a large effect size). Two-way
243 ANOVA was used to analyze the main effects of genotype and experience on male sexual
244 behavior and lordosis quotient or genotype and stimulus on olfactory preference. Tukey post-
245 hoc tests were used to determine group differences. Effect sizes were further estimated by
246 calculating the eta-squared η^2 ($\eta^2 = SS_{\text{effect}} / SS_{\text{total}}$, where SS_{effect} is the sums of squares for the
247 effect of interest and SS_{total} is the total sums of squares for all effects, interactions and errors,
248 $\eta^2 = 0.02$ is considered as a small effect size, $\eta^2 = 0.13$ as a medium effect size and $\eta^2 = 0.26$
249 as a large effect size). As variances were not homogeneous between groups, TH-, kisspeptin-
250 ER α - and AR immunoreactivity was analyzed with Mann-Whitney nonparametric test. P
251 values of less than 0.05 were considered to be significant.

252

253 **Results**

254 **General characterization of the $ER\beta^{NesCre}$ mouse line**

255 The selective neural deletion of $ER\beta$ was confirmed by RT-PCR. A 177 bp-amplified
256 fragment was present at comparable levels of expression in the epididymis of $ER\beta^{fl/fl}$ and
257 $ER\beta^{NesCre}$ males ($t = -2.543$, $p = 0.064$, $d = -2.540$). This signal was present in the brain of
258 $ER\beta^{fl/fl}$ males, and highly reduced in their $ER\beta^{NesCre}$ littermates (-98%, $t = 7.716$, $p = 0.001$, d
259 $= 7.660$; Fig. 1A-B). The NesCre transgene used triggers gene deletion in neural precursor
260 cells by embryonic day 10.5, before gonadal differentiation. To ensure that excision of neural
261 $ER\beta$ exon 3 was indeed efficient during the perinatal organization of the male brain, we
262 performed a PCR analysis for $ER\beta$ alleles and Cre recombinase on DNA extracted from
263 neonatal and adult brains. A small amplicon of 250 bp indicating Cre-mediated excision of
264 $ER\beta$ exon 3 was found in the brain of both neonatal and adult males expressing the Cre
265 recombinase (Fig. 1C). By comparison, a 850 bp signal corresponding to the floxed allele was
266 seen in the brain of control littermates lacking the NesCre transgene.

267 Student's t tests showed no significant effect of the $ER\beta^{NesCre}$ mutation on circulating levels of
268 testosterone ($t = 0.731$, $p = 0.470$, $d = 0.250$ versus $ER\beta^{fl/fl}$; Fig. 1D). This result was
269 corroborated by the unchanged weight of the androgen-dependent seminal vesicles in
270 $ER\beta^{NesCre}$ males ($t = -1.312$, $p = 0.198$, $d = -0.650$ versus controls; Fig. 1E). In fertility tests,
271 $ER\beta^{NesCre}$ males produced a total number of 45 ± 5.0 pups versus 46 ± 3.0 for $ER\beta^{fl/fl}$ mice (t
272 $= 0.083$, $p = 0.936$, $d = 0.070$). The interval from mating to first litter was also similar
273 between the two genotypes (22 ± 0.4 days in $ER\beta^{NesCre}$ versus 21 ± 1.0 days in $ER\beta^{fl/fl}$; $t =$
274 0.600 , $p = 0.570$, $d = 0.490$).

275

276 **Behavioral effects of neural $ER\beta$ invalidation**

277 *Male sexual behavior and olfactory preference of intact males*

278 Comparable percentages of naïve males (86% of $ER\beta^{fl/fl}$ and 81% of $ER\beta^{NesCre}$ genotypes)
279 displayed a full range of sexual behavior and reached ejaculation. Analyses of the latencies to
280 the first behaviors showed a significant effect of experience for the latencies to the first mount
281 ($F_{(1,28)} = 11.240$, $p = 0.002$, $\eta^2 = 0.141$), pelvic thrusting ($F_{(1,28)} = 12.400$, $p = 0.001$, $\eta^2 =$
282 0.165), intromission ($F_{(1,28)} = 13.450$, $p = 0.002$, $\eta^2 = 0.156$) and latency to ejaculation ($F_{(1,28)}$
283 $= 14.730$, $p < 0.001$, $\eta^2 = 0.186$) as illustrated in Fig. 2A. There was no significant effect of
284 genotype on the latencies of these behaviors (mount: $F_{(1,28)} = 2.100$, $p = 0.160$, $\eta^2 = 0.035$;
285 intromission: $F_{(1,28)} = 2.650$, $p = 0.115$, $\eta^2 = 0.042$; thrusting: $F_{(1,28)} = 2.890$, $p = 0.100$, $\eta^2 =$
286 0.046 ; ejaculation: $F_{(1,28)} = 2.960$, $p = 0.096$, $\eta^2 = 0.043$). The number of mounts without (M)
287 or with intromission (MI), the total number of thrusts and mating length for both naïve and
288 experienced males were also quantified (Table 1). Two-way ANOVA showed a significant
289 effect of experience for the number of thrusts ($F_{(1,28)} = 6.920$, $p = 0.014$, $\eta^2 = 0.105$) and
290 mating length ($F_{(1,28)} = 4.520$, $p = 0.042$, $\eta^2 = 0.070$) but not of genotype ($F_{(1,28)} = 0.610$, $p =$
291 0.441 , $\eta^2 = 0.010$ and $F_{(1,28)} = 0.710$, $p = 0.408$, $\eta^2 = 0.012$, respectively). There was no
292 significant effect of experience (M: $F_{(1,28)} = 0.250$, $p = 0.622$, $\eta^2 = 0.005$; MI: $F_{(1,28)} = 0.850$, p
293 $= 0.360$, $\eta^2 = 0.012$) or genotype (M: $F_{(1,28)} = 0.070$, $p = 0.799$, $\eta^2 = 0.001$; MI: $F_{(1,28)} = 1.410$,
294 $p = 0.240$, $\eta^2 = 0.027$) on the other components of mating.

295 The ability of males to discriminate between male and female pheromones in tests using
296 gonad-intact male versus estrus female was tested. There was a significant effect of stimulus
297 ($F_{(1,25)} = 7.020$, $p = 0.023$, $\eta^2 = 0.211$) but not of genotype ($F_{(1,25)} = 0.350$, $p = 0.565$, $\eta^2 =$
298 0.011 ; Fig. 2B). The total time devoted to chemoinvestigation was not significantly different
299 between sexually experienced $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males (128.06 ± 10.35 sec versus 150.38
300 ± 12.93 sec, respectively; $t = 0.601$, $p = 0.560$, $d = 0.360$).

301

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

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

316 The males were then subjected to olfactory preference tests. There was no significant effect of
317 stimulus ($F_{(1,33)} = 0.050, p = 0.825, \eta^2 = 0.002$) or genotype ($F_{(1,33)} = 1.210, p = 0.290, \eta^2 =$
318 0.033), indicating that males of the two genotypes displayed no olfactory preference (Fig.
319 2D). The total time spent investigating the two cues was similar between the two genotypes
320 (117.97 ± 9.03 sec versus 106.99 ± 4.98 sec, respectively; $t = 1.098, p = 0.290, d = 0.570$).

321

322 *Neuroanatomical organization of the medial preoptic area*

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

327 assessed whether neural *ERβ* invalidation alters the neuronatomical organization of TH-
328 immunoreactive cells. In accordance with previous studies (Simerly et al., 1985), the number
329 of TH-immunoreactive cells was greater (2.4-fold) in females than in $ER\beta^{fl/fl}$ males ($p =$
330 0.034; Fig. 3A and C). $ER\beta^{NesCre}$ males showed a male pattern since no significant differences
331 were seen with their $ER\beta^{fl/fl}$ littermates ($p = 0.885$). The number of kisspeptin-
332 immunoreactive cells, another sexually dimorphic population, was then quantified in the three
333 subdivisions of the rostral periventricular area of the third ventricle (RP3V). Data show sex
334 differences, with females exhibiting 24 to 225-fold higher number of kisspeptin-ir neurons in
335 the AVPV ($p = 0.026$), rostral ($p = 0.034$) and caudal ($p = 0.028$) periventricular nuclei than
336 $ER\beta^{fl/fl}$ males (Fig. 3B and D). Again, no significant differences were observed between
337 $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males ($p = 0.317$, $p = 0.102$ and $p = 0.278$ for the AVPV, rPeN and
338 cPeN, respectively; Fig. 3D).

339

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

341 *ERα* and AR signaling pathways play an important role in the expression of male sexual
342 behavior. It was thus evaluated whether neural *ERβ* invalidation altered the number of *ERα*-
343 and AR-immunoreactive cells in the neural circuitry underlying this behavior. The number of
344 *ERα* -immunoreactive cells was unchanged in the MeA ($p = 0.275$) and MPOA ($p = 0.513$) of
345 $ER\beta^{NesCre}$ males by comparison to $ER\beta^{fl/fl}$ males (Fig. 4A-B). It was, however, significantly
346 increased by 37% in the BNST ($p = 0.050$ versus $ER\beta^{fl/fl}$ genotype). Similarly, the number of
347 AR-immunoreactive cells was unaltered in the MeA ($p = 0.827$) and MPOA ($p = 0.513$) and
348 significantly increased in the BNST of $ER\beta^{NesCre}$ mice (+38%, $p = 0.050$ versus $ER\beta^{fl/fl}$
349 genotype; Fig. 5A-B).

350

351 **Discussion**

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

356 In naïve $ER\beta^{NesCre}$ males, the latencies and frequencies to perform the various components of
357 copulatory behavior were not statistically different from those observed in their control
358 littermates. Sexual experience ameliorated mating in both $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males by
359 reducing the latencies to behaviors and mating length, and by increasing the number of
360 thrusts. $ER\beta^{NesCre}$ males exhibited also normal preference towards female olfactory cues.

361 These results are in agreement with the lack of *ERβ* involvement in the masculinization of
362 sexual behavior and olfactory preference previously reported for the initial global *ERβ*
363 knockout model (Kudwa et al., 2005; Ogawa et al., 1999). They contrast with the recent
364 global *ERβ* invalidation (Antal et al., 2008), which resulted in increased number of mounts
365 and intromissions and delayed ejaculation, although sexual experience progressively restored
366 these behavioral differences (Antal et al., 2012). As these mice were obtained from the same
367 floxed model and similar genetic background as the present conditional model, we suggest
368 that the mild behavioral deficiency induced by this global mutation was probably due to
369 peripheral effects of *ERβ* deletion. The present conditional mutation did not alter male
370 fertility and circulating levels of testosterone while the global *ERβ* deletion generated by
371 Chambon's laboratory resulted in an infertile phenotype of unknown origin (Antal et al.,
372 2008). Whether or not these global *ERβ* knockout males exhibit altered regulation of the
373 hypothalamus-pituitary-gonad axis, which may in turn interfere with reproductive behaviors,
374 has not been reported. In males, *ERβ* is expressed in somatic and germ cells of the testis (van
375 Pelt et al., 1999) and seems to be involved in testosterone production (Dumasia et al., 2015).

376

377 It has been shown that *ERβ* is important in the defeminization processes induced
378 perinatally by estradiol in the neural circuitry underlying male sexual behavior (Kudwa et al.,
379 2005). Mutant males from the initial global *ERβ* knockout model (Krege et al., 1998),
380 exhibited a higher lordosis quotient than the wild-type males, after adult castration and
381 priming with estradiol and progesterone. In our conditional model, only a small percentage of
382 $ER\beta^{NesCre}$ males castrated and primed with estradiol and progesterone, showed a lordosis
383 posture. Furthermore, $ER\beta^{NesCre}$ males exhibiting lordosis behavior displayed a low LQ (5%)
384 in Test 3 while a mean value of 25% was reported for global *ERβ* knockout mice (Kudwa et
385 al., 2005). Moreover, this low expression of lordosis behavior by $ER\beta^{NesCre}$ males was random
386 across tests, indicating that there was no effect of sexual experience. In similar conditions, a
387 group of control females exhibited a high LQ reaching 71% since Test 2. This demonstrates
388 that the low behavior displayed by $ER\beta^{NesCre}$ males was not due to experimental limitations.

389 At the neuroanatomical level, the number of TH-ir neurons was not modified in the AVPV of
390 $ER\beta^{NesCre}$ males. These results contrast with previous studies showing increased number of
391 TH-ir neurons in the AVPV of global *ERβ* knockout males (Bodo et al., 2006). However,
392 analysis of kisspeptin neurons, another sexually dimorphic population of the RP3V,
393 confirmed the lack of $ER\beta^{NesCre}$ mutation effect on the sexual differentiation of the AVPV.

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

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

406

407 Neural invalidation of *ERβ* resulted in increased number of AR- and *ERα*-immunoreactive
408 neurons specifically in the BNST. Previous studies reported that *ERβ* modulates *ERα*
409 expression in hypothalamic cells (Malikov and Madeira, 2013). Whether *ERβ* modulates the
410 expression of both AR and *ERα* in the BNST needs further investigation. Nevertheless, such
411 cross-regulations between sex steroid receptor signaling pathways are not uncommon since a
412 similar increase of *ERα*-immunoreactive cell number was noticed in the MeA and MPOA of
413 males lacking neural AR (Picot et al., 2014). It is unlikely that the increased amount of AR
414 and *ERα* proteins in the BNST compensates for the lack of *ERβ* in the expression of sexual
415 behavior. Indeed, *ERβ* was deleted along the neural circuitry underlying sexual behavior and
416 no changes in AR- or *ERα*-immunoreactivity were observed in the MeA or MPOA. The
417 BNST is involved in other behaviors such as anxiety-like behavior (Daniel and Rainnie,
418 2015). Administration of a selective *ERβ* agonist to ovariectomized female rats has an
419 anxiolytic effect (Lund et al., 2005; Weiser et al., 2009). This anxiolytic-like effect was
420 observed in wild-type female mice but not in global *ERβ* knockouts (Oyola et al., 2012; Walf
421 et al., 2008), which exhibit increased anxiety-like behavior (Krezel et al., 2001). In agreement
422 with these observations, neural deletion of *ERβ* results in increased anxiety-state level during
423 the follicular phase in female mice (Naulé et al., 2015). In male mice, the involvement of *ERβ*
424 in estrogen-modulated anxiety state still needs to be documented. Minor effects of gene

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

430

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

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

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

462

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

471

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478

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628

629 **Figure legends**

630 **Fig. 1. Characterization of the $ER\beta^{NesCre}$ mouse line.** (A) RT-PCR of total RNAs obtained
631 from the brain (Br) and epididymis (Ep). The representative gel shows the presence of the 177
632 base pair (bp) amplified fragment in the epididymis of both genotypes and only in the brain of
633 control $ER\beta^{fl/fl}$ males. DNA size markers at 50 bp increments are shown in the left column.
634 (B) Quantitative data normalized to GAPDH from 3 males per genotype. $**p < 0.01$ versus
635 $ER\beta^{fl/fl}$ brain. (C) PCR analyses performed on the brain of three neonates and four adults,
636 obtained from the same litters, respectively. Up: PCR analysis showing the presence of the
637 floxed $ER\beta$ allele (850 bp) in the neonatal and adult brain of $ER\beta^{fl/fl}$ mice (2, 3, 5, 7). The
638 small amplicon of 250 base pair (bp) indicating Cre-mediated excision of exon 3 was present
639 in the neonatal and adult brain of $ER\beta^{NesCre}$ littermates (1, 4, 6). Down: PCR analysis showing
640 the presence of Cre recombinase in the neonatal and adult brain of $ER\beta^{NesCre}$ mice expressing
641 the excised $ER\beta$ allele (1, 4, 6). DNA size markers at 100 bp increments are shown in the left
642 column. (D) Circulating levels of testosterone in $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males (n = 10 per
643 genotype). (E) Weight of seminal vesicles (SV) expressed as percentage of body weight (bw)
644 in male mice (n = 10 per genotype).

645

646 **Fig. 2. Effects of neural $ER\beta$ invalidation on sexual behavior and olfactory preference in**
647 **males.** (A) Latencies to the first mount (Mo), thrust (Th), intromission (In), and ejaculation
648 (Ej) of intact $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males in Tests 1 and 2 (n = 13-17 animals per genotype).
649 $^a p < 0.05$ versus Test 2. (B) Time spent chemoinvestigating gonad-intact male (M) versus
650 estrus female (F) expressed as percentage of the total time chemoinvestigating (n = 9-10
651 males per genotype). $^a p < 0.05$ versus female stimulus. (C) Lordosis quotient of castrated
652 males supplemented with estradiol and progesterone in three successive tests (n = 6-10 males
653 per genotype). (D) Time spent chemoinvestigating intact males (M) versus estrus females (F),

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

656

657 **Fig. 3. Tyrosine hydroxylase (TH) and kisspeptin immunoreactivity in $ER\beta^{fl/fl}$ and**
658 **$ER\beta^{NesCre}$ males. (A-B)** Representative immunostaining of TH (A) and kisspeptin (B) in
659 $ER\beta^{fl/fl}$ males, their mutant littermates ($ER\beta^{NesCre}$) males and in control females. Scale bar =
660 100 μ m. (C-D) Quantitative data for TH- in the anteroventral periventricular nucleus (AVPV)
661 (C) and kisspeptin-immunoreactivity in the AVPV, rostral (rPeN) and caudal (cPeN)
662 periventricular nuclei (D) are expressed as mean values \pm S.E.M for 4 animals per group. ^a $p <$
663 0.05 versus $ER\beta^{fl/fl}$ males.

664

665 **Fig. 4. Quantification of $ER\alpha$ -immunoreactive cell number in brain areas of $ER\beta^{fl/fl}$ and**
666 **$ER\beta^{NesCre}$ males. (A)** Representative anti- $ER\alpha$ immunostaining in the medial amygdala
667 (MeA), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) of
668 $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males. Scale bar = 100 μ m. AC, anterior commissure. (B) Quantitative
669 data for the number of $ER\alpha$ -immunoreactive (ir) cells are expressed as mean values \pm S.E.M
670 for 3-4 animals per genotype. ^a $p <$ 0.05 versus $ER\beta^{fl/fl}$ males in the BNST.

671

672 **Fig. 5. Quantification of AR-immunoreactive cell number in brain areas of $ER\beta^{fl/fl}$ and**
673 **$ER\beta^{NesCre}$ males. (A)** Representative anti-AR immunostaining in the medial amygdala
674 (MeA), bed nucleus of the stria terminalis (BNST) and medial preoptic area (MPOA) of
675 $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males. Scale bar = 100 μ m. AC, anterior commissure. (B) Quantitative
676 data for the number of AR-immunoreactive (ir) cells are expressed as mean values \pm S.E.M
677 for 3-4 animals per genotype. ^a $p <$ 0.05 versus $ER\beta^{fl/fl}$ males in the BNST.

678 **Table legends.**

679 **Table 1. Quantification of the sexual behavior displayed by naïve and sexually**
680 **experienced $ER\beta^{fl/fl}$ and $ER\beta^{NesCre}$ males.** The number of mounts without (M) or with
681 intromission (MI), the total number of thrusts (Th) and mating length are shown for males (n
682 = 13-17 per genotype) tested in Tests 1 and Test 2. ^a $p < 0.05$ versus Test 1.
683

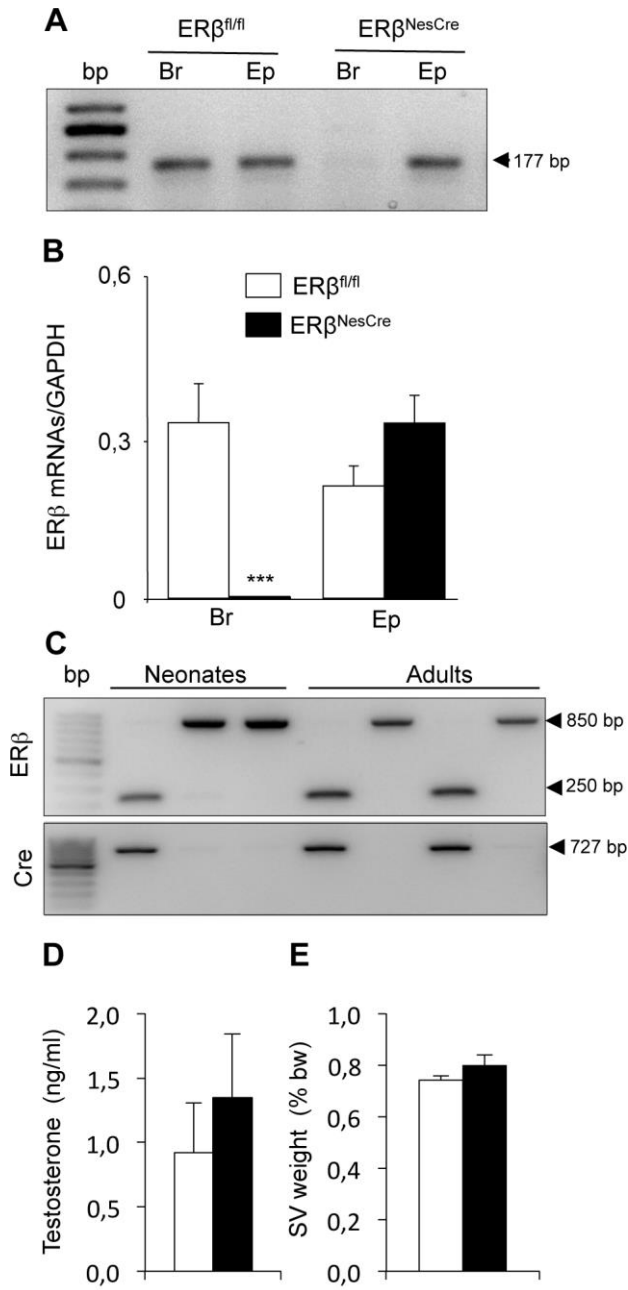
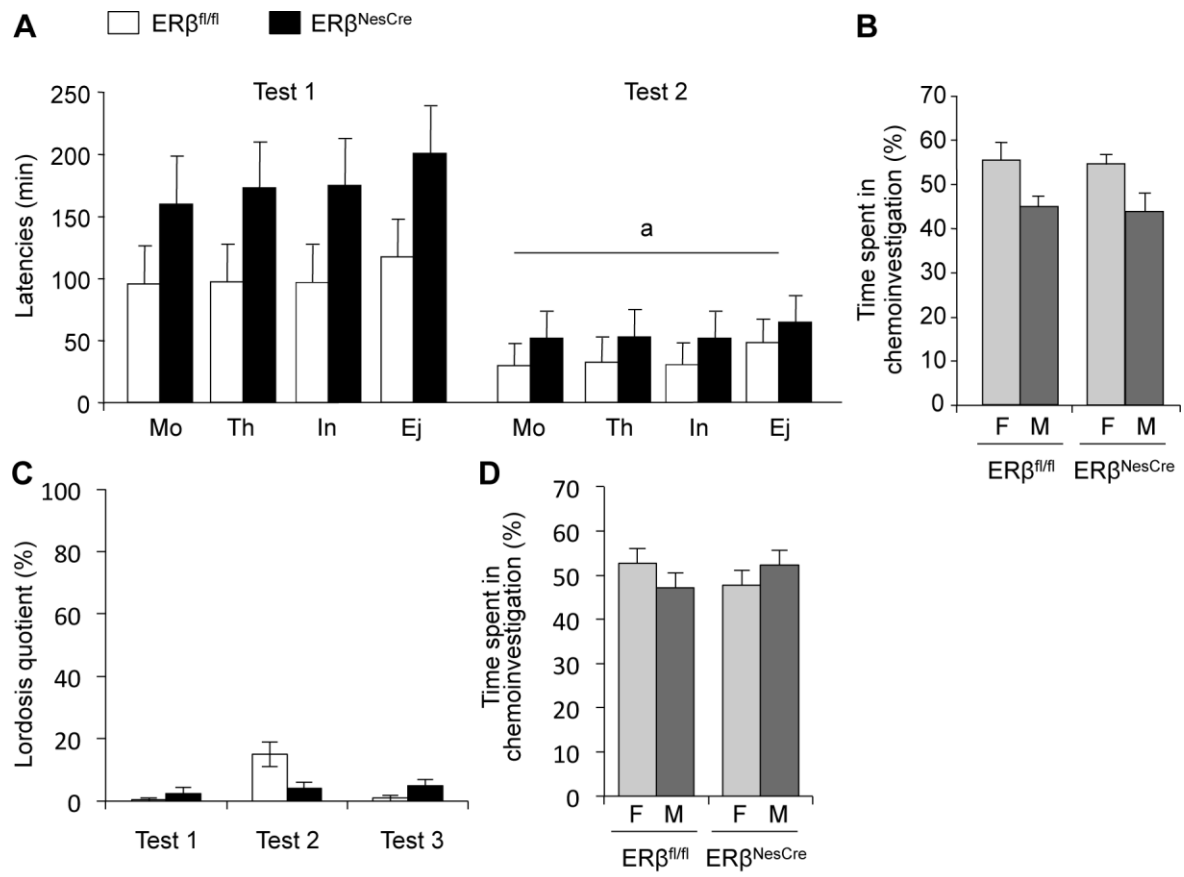


Figure 1

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Figure 2

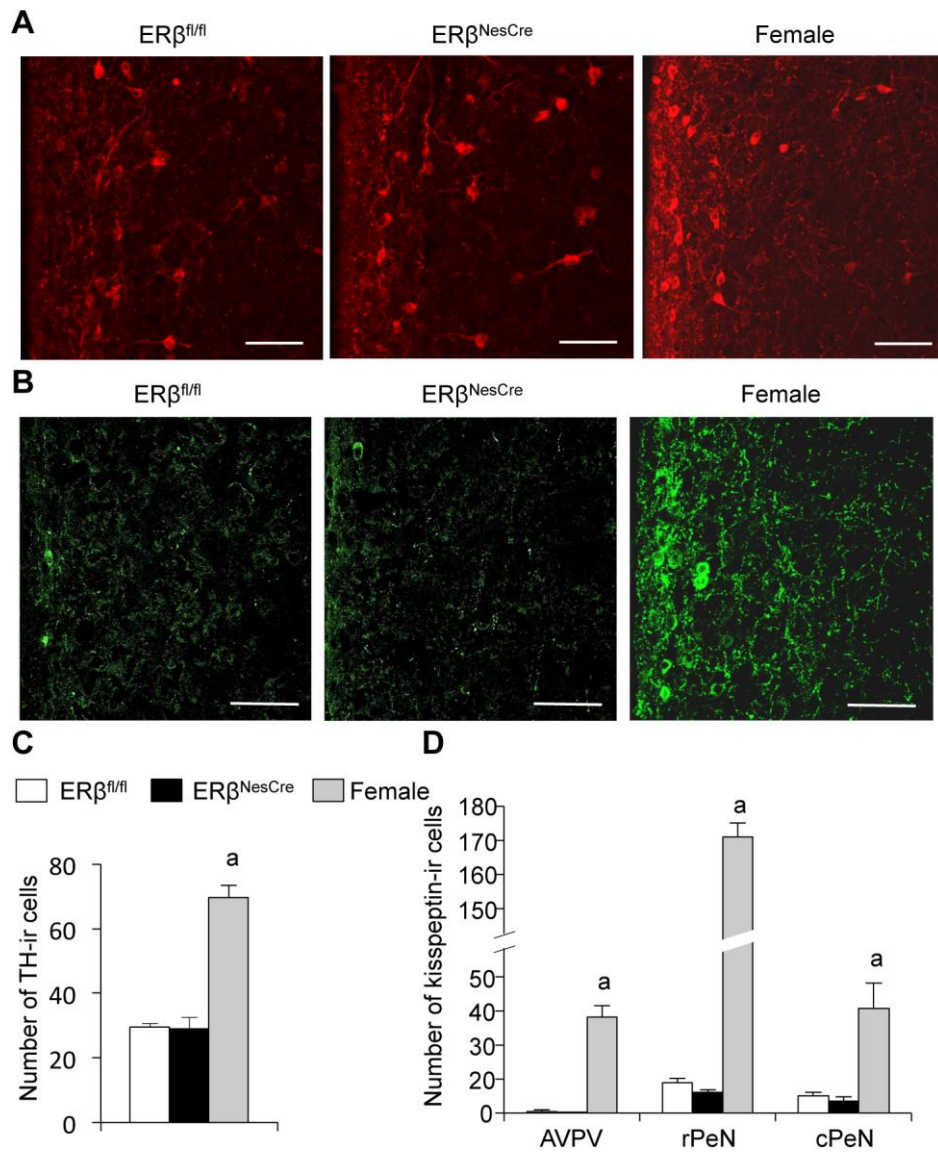


Figure 3

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689

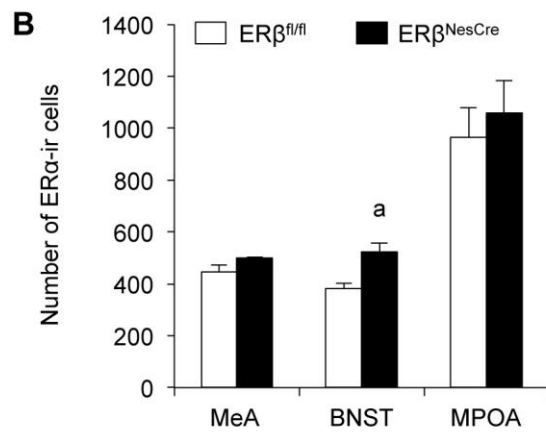
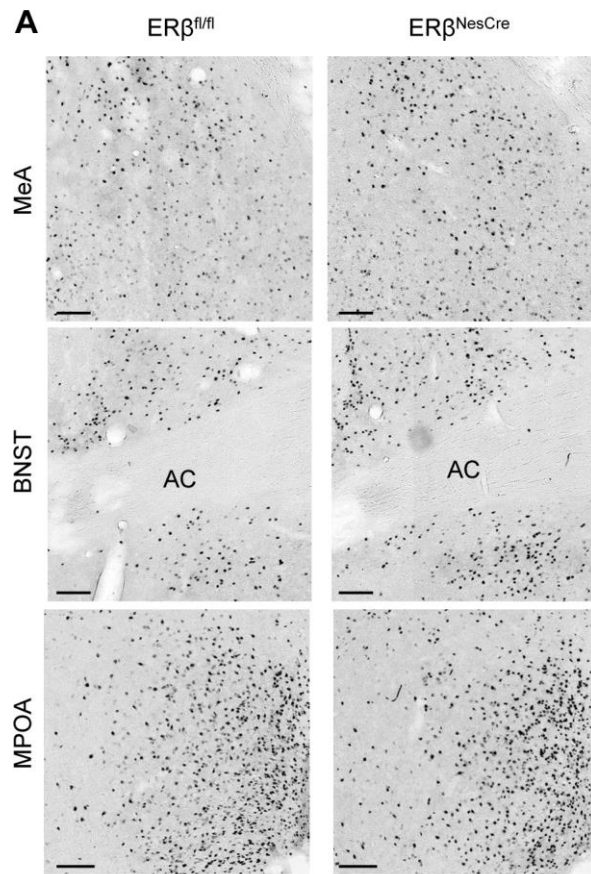


Figure 4

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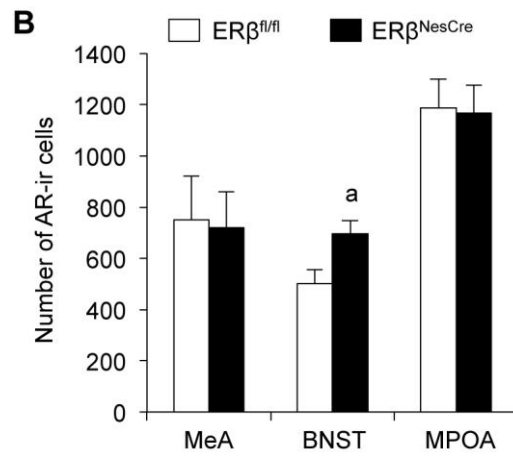
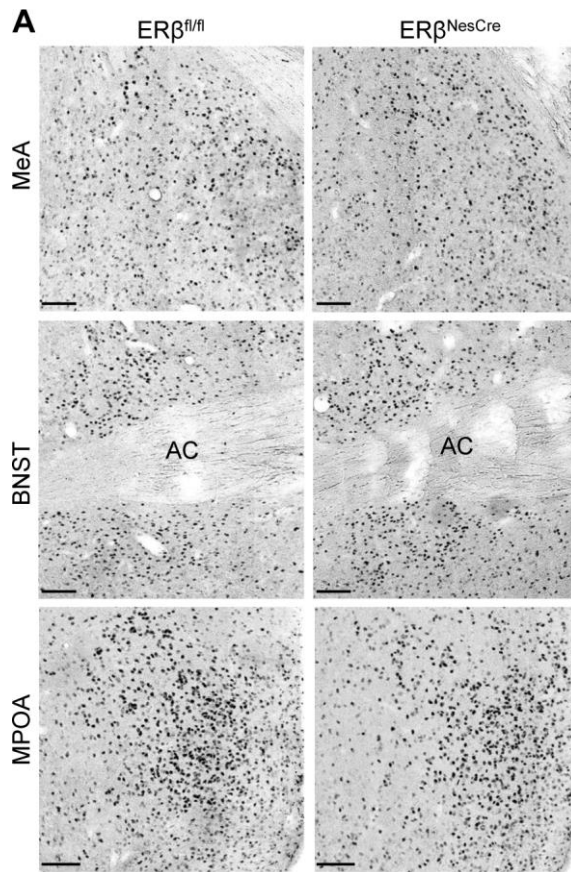


Figure 5