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1 **Title:** Deletion of neural estrogen receptor alpha induces sex differential effects on reproductive  
2 behavior in mice

3

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21

22 **Abstract**

23 Estrogen receptor (ER)  $\alpha$  is involved in several estrogen-modulated neural and peripheral functions.  
24 To determine its role in the expression of female and male reproductive behavior, a mouse line  
25 lacking the *ER $\alpha$*  in the nervous system was generated. Mutant females did not exhibit sexual  
26 behavior despite normal olfactory preference, and had a reduced number of progesterone receptor-  
27 immunoreactive neurons in the ventromedial hypothalamus. Mutant males displayed a moderately  
28 impaired sexual behavior and unaffected fertility, despite evidences of altered organization of  
29 sexually dimorphic populations in the preoptic area. In comparison, males deleted for both neural  
30 *ER $\alpha$*  and androgen receptor (*AR*) displayed greater sexual deficiencies. Thus, these data highlight a  
31 predominant role for neural *ER $\alpha$*  in females and a complementary role with the *AR* in males in the  
32 regulation of sexual behavior, and provide a solid background for future analyses of neuronal versus  
33 glial implication of these signaling pathways in both sexes.

34

## 35 **Introduction**

36 Sex steroids play a key role in the developmental organization and adult activation of the neural  
37 circuitry underlying reproductive behavior. In males, it is largely accepted that perinatal  
38 testosterone and its neural metabolite estradiol irreversibly masculinize and defeminize the neural  
39 structures underlying behavioral responses [1]. During this period, the ovaries are inactive and the  
40  $\alpha$ -fetoprotein protects the female brain from these masculinizing effects by selectively binding to  
41 maternal and male sibling-derived estradiol [2]. The ovaries start secreting estradiol around  
42 postnatal day 7 [3]. It has been suggested that estradiol, during the time window between postnatal  
43 days 15 and 25, is important for a full expression of female sexual behavior during adulthood [4, 5].

44  
45 In the nervous system, testosterone activates the androgen receptor (AR), while estradiol acts  
46 mainly through two nuclear estrogen receptors (ER)  $\alpha$  and  $\beta$  encoded by two different genes, with  
47 increasing evidence indicating the potential involvement of other non-genomic pathways as well  
48 [6]. Conditional genetic mouse models were generated to determine the neural role of nuclear AR  
49 and ERs in the expression of reproductive behavior, without interfering with their peripheral  
50 functions. We have previously shown that early *AR* deletion in neuronal and glial cells impaired the  
51 expression of sexual and aggressive behaviors in males [7-8]. In particular, neural *AR* deletion did  
52 not affect the perinatal organization of known sexually dimorphic populations in the preoptic area,  
53 the key hypothalamic region controlling male sexual behavior [9-10], but was found to alter the  
54 postnatal organization of the spinal nuclei involved in erection and ejaculation [11-12]. Further  
55 neural deletion of *ER $\beta$* , using the same transgene, showed that this receptor is not involved in the  
56 expression of male or female sexual behavior, but rather mediates estrogen-induced regulation of  
57 social and aggressive behaviors in males and mood-related behavior in both sexes [13-15]. In this  
58 context, the reproductive phenotype induced by early neural *ER $\alpha$*  deletion using this same transgene  
59 still needs to be determined. Previous studies addressing the effects of *ER $\alpha$*  deletion in neurons [16-  
60 17], or in specific neuronal populations such as glutamatergic and GABAergic neurons [18], or

61 Tac2 or kisspeptin neurons [19-22] only focused on the regulation of the hypothalamic-pituitary-  
62 gonadal axis in females. In a male study, it has been shown that postnatal *ERα* deletion in vesicular  
63 GABA transporter (Vgat) neurons altered mating and territorial behaviors without changing  
64 hormonal levels [23], while its deletion in vesicular glutamate transporter (Vglut) 2 neurons  
65 increased testosterone levels but did not affect these behaviors. Other studies conducted on males  
66 targeted life stages after the perinatal period. A study using viral-mediated knockdown of *ERα* in  
67 specific brain areas reported that pubertal *ERα* activation in the medial amygdala is crucial for male  
68 behavior expression, while adult *ERα* expression in the medial preoptic and ventromedial nuclei  
69 facilitates this behavior [24-25].

70

71 The present study was therefore conducted to investigate the effects of neural *ERα* deletion on  
72 male and female behaviors. This mouse model was generated using the nestin-Cre transgene, which  
73 triggers gene deletion in neural precursor cells by embryonic day 10.5, before gonadal  
74 differentiation, as previously reported for neural deletions of *AR* and *ERβ* [8, 14]. For this purpose,  
75 the impact of *ERα*<sup>NesCre</sup> mutation was assessed in males and females on reproductive parameters  
76 including hormonal levels, weight of androgen- or estrogen-dependent organs, sperm count,  
77 presence of corpora lutea and fertility. We also assessed the behaviors related to reproduction  
78 (mating, olfactory preference, emission of ultrasonic vocalizations), locomotor activity, anxiety-like  
79 behavior and aggression. The neuroanatomical organization of sexually dimorphic hypothalamic  
80 populations related to male or female sexual behaviors were analyzed. Finally, a double neural  
81 knockout for *AR* and *ERα* (*AR::ERα*<sup>NesCre</sup>) was generated and the behavioral effects of deletion of  
82 both receptors were analyzed in males.

83

## 84 **Results**

### 85 **Selective neural *ERα* deletion and physiological parameters in males and females**

86 The selective neural deletion of *ERα* is shown by the presence of the deleted *ERα* allele of 223 bp  
87 in the brain but not in the ovary of floxed mice expressing the Cre recombinase (Figure 1a and  
88 Supplementary Figure 1). RT-PCR analysis showed comparable *ERα* mRNA levels in the ovary  
89 and testis of control and mutant animals and reduced levels in the cortex, hippocampus and  
90 hypothalamus of mutant females (-71%, -71% and -78%, respectively) and males (-75%, -87% and  
91 -84%, respectively) in comparison with their control littermates (Figures 1b-c).

92 Mutant females were found to have unchanged body weight, reduced relative weight of ovaries (-  
93 25%) and a higher relative weight of the uterus (+170%), an estrogen-sensitive tissue, as compared  
94 with the control group (Supplementary Table 1). In agreement with the latter result, circulating  
95 levels of estradiol were significantly increased in mutant females (+61% versus controls).  
96 Histological analysis showed no corpora lutea in the ovaries of mutant females (Supplementary  
97 Table 1). In mutant males, body weight was reduced (-5.5%). The relative weight of the seminal  
98 vesicles, which are androgen-sensitive, and testosterone levels were increased (+20% and +100%,  
99 respectively), while estradiol levels were reduced (-39%) in comparison with control mice  
100 (Supplementary Table 1). The other reproductive parameters, such as testis weight or epididymal  
101 sperm count, were unchanged between the two genotypes.

102

### 103 **Arrested estrous cycle and abolished sexual behavior in neural *ERα* knockout females**

104 Analysis of the estrous cycle over three weeks showed that mutant females spent more time in the  
105 metestrous stage (+174%) and less time (-70 to -93%) in the other stages in comparison with  
106 controls (Figure 2a). The estrous cycle arrest was associated with a reduced number of kisspeptin-  
107 immunoreactive neurons in the anteroventral periventricular area (AVPV) of mutant females (-78%  
108 versus controls at the proestrus-estrus and -68% versus controls at the metestrus-diestrus) (Figure  
109 2b-c). For the other sexually dimorphic population of the AVPV, the number of tyrosine

110 hydroxylase (TH)-expressing neurons is shown for control females regardless of their estrous stage  
111 (Figure 2d-e) since no significant differences were seen between the metestrus-diestrus and  
112 proestrus-estrus stages as previously reported [26-27]. Neural *ERα* deletion reduced by 46% the  
113 number of TH-immunoreactive cells in mutant females in comparison with controls (Figure 2e).  
114 Behavioral analyses were performed on ovariectomized females primed with estradiol and  
115 progesterone to induce their receptivity under comparable hormonal conditions. Mutant females  
116 never exhibited lordosis posture in response to male mounts over three tests, while their control  
117 littermates displayed an increased lordosis quotient over tests reaching 0.7 at the last test (Figure  
118 2f). This alteration was not due to an effect of neural *ERα* deletion on olfactory preference since  
119 females investigated males more than females regardless of their genotype (Figure 2g). The  
120 impaired lordosis behavior of mutant females was associated with a lower number of progesterone  
121 receptor (PR)-immunoreactive neurons (-84% versus controls) in the ventromedial hypothalamus as  
122 shown in Figure 2h.

123

#### 124 **Neural *ERα* deletion reduced but did not abolish male sexual behavior**

125 All males initiated and achieved sexual behavior in both tests 1 and 2 regardless of their genotype  
126 (Figure 3a). However, differences between controls and mutants were seen in the latency to initiate  
127 mounting, intromissions and to reach ejaculation at both tests (Figure 3b). In accordance, the mating  
128 duration was higher in mutant males (+287% and +420% in Tests 1 and 2, respectively) as shown in  
129 Figure 3c. Quantification of the behavioral events showed that mutant males exhibited more mount  
130 attempts with or without intromissions and pelvic thrusts (1.8- to 2.4-fold above controls) in both  
131 tests (Table 1). These findings together with the increased interval between two consecutive  
132 intromissions (+85% and +265% above controls at tests 1 and 2, respectively) suggest a lower  
133 behavior of mutant males.

134 In the olfactory preference test, there was an effect of stimulus but not of genotype, with all males  
135 spending more time investigating the female stimulus (Figure 3d). Analysis of the emission of

136 courtship ultrasonic vocalizations in the presence of a sexually receptive female showed a lower  
137 number (-21%) and duration (-30%) of ultrasonic vocalizations in mutant males in comparison with  
138 the control group (Figure 3e-f, h). Detailed quantification of the nine emitted syllables showed a  
139 reduced number and duration of the complex, mixed and frequency-jump syllables (Figure 3g, i).

140 At the neuroanatomical level, the effects of neural *ERα* deletion were investigated in two sexually  
141 dimorphic neuronal populations known to be organized by perinatal estradiol in the medial preoptic  
142 area [28-29]. The number of calbindin-immunoreactive neurons was reduced (-40%; Figure 4a-b),  
143 while that of TH-immunoreactive neurons was higher (+80%; Figure 4c-d) in mutant males  
144 compared to their control littermates. Immunohistochemical analysis of the AR showed a  
145 comparable number of AR-immunoreactive neurons in control and mutant males in the medial  
146 amygdala, bed nucleus of stria terminalis, medial preoptic area and lateral septum (Figure 4e-f).

147 The unchanged number of AR-immunoreactive neurons in key regions underlying sexual behavior,  
148 together with the increased levels of circulating testosterone may compensate for the sexual  
149 dysfunction caused by neural *ERα* deletion. We thus analyzed sexual behavior of control and  
150 mutant males, which were gonadectomized and supplemented with equivalent levels of  
151 testosterone. Table 2 shows that under these normalized hormonal conditions, evidenced by the  
152 comparable relative weight of seminal vesicles, the latencies to initiate the first mount and  
153 intromission and to reach ejaculation were higher in sexually experienced mutants (7.2-, 6- and 3.5-  
154 fold above controls, respectively). In contrast, no differences were found between the two  
155 genotypes in the number of mounts, intromissions and pelvic thrusts.

156 In continuous fertility tests with control females, neural *ERα* knockout males were able to produce  
157 offspring. The latency to produce the first litter ( $20.33 \pm 0.33$  days versus  $27.67 \pm 0.67$  days for  
158 controls), the number of litters ( $7.33 \pm 0.29$  versus  $7.33 \pm 0.33$  for controls) and litter size ( $6.26 \pm$   
159  $0.93$  versus  $6.77 \pm 0.76$  for controls) were comparable between the two genotypes.

160

161 **Double neural AR and *ERα* knockout severely impaired male sexual behavior in mice**



162 In previous studies, we have shown that AR<sup>NesCre</sup> deletion greatly impaired but did not abolish the  
163 expression of male sexual behavior [8, 11]. We thus determined the impact of neural deletion of  
164 both *AR* and *ERα* on male behaviors. In this double knockout model, only 57% of AR::*ERα*<sup>NesCre</sup>  
165 mutant males versus 100% of controls reached ejaculation in tests 1 and 2 (Figure 5a). In addition,  
166 the latencies to initiate the first mount and intromission and to reach ejaculation were greatly  
167 increased in test 1 for mutants (65-fold, 58-fold and 14-fold above controls, respectively) (Figure  
168 5b). There was no significant amelioration in Test 2 (48-fold, 51-fold and 24-fold above controls,  
169 respectively). In accordance, mating duration was significantly increased in both Tests 1 and 2 (12-  
170 fold and 28-fold above controls, respectively) (Figure 5c). Quantification of the behavioral events  
171 showed reduced number of intromissions and thrusts in test 1, a higher number of mounts in test 2  
172 and a reduced intromission ratio and a higher intromission interval for mutants in both tests (Table  
173 3).

174 This sexual alteration was associated with a normal olfactory preference towards female stimuli  
175 (Figure 5d) but a reduced total number and duration of emitted syllables (-43% and -51 % versus  
176 controls, respectively) (Figure 5e).

177 Double neural AR::*ERα*<sup>NesCre</sup> males had a lower body weight (-15%) and a higher relative weight of  
178 seminal vesicles (+53%) and circulating levels of testosterone (+100%) in comparison with their  
179 control littermates (Supplementary Table 2).

180

### 181 **Effects of neural *ERα* or AR::*ERα* deletion on other behaviors**

182 Male and female mice, subjected to sexual behavior tests, were also analyzed for locomotor activity  
183 and anxiety-state level. These latter behaviors are also regulated by estrogens and could  
184 interfere with the expression of sexual behavior if altered. Ovariectomized and hormonally primed  
185 females carrying the *ERα*<sup>NesCre</sup> mutation and gonadally-intact *ERα*<sup>NesCre</sup> males displayed a  
186 comparable activity to their respective control littermates in the circular corridor over the 120-min  
187 test (Figure 6a, c). Similarly, no differences were found between control and mutant *ERα*<sup>NesCre</sup>

188 males, which were castrated and supplemented with equivalent testosterone concentrations  
189 (Supplementary Figure 2a). In contrast, a higher locomotor activity (+44%) was observed in intact  
190 males carrying the double  $AR::ER\alpha^{NesCre}$  mutation compared to their control littermates (Figure 6e).  
191 The anxiety-state level assessed in the O-Maze was increased in  $ER\alpha^{NesCre}$  females as evidenced by  
192 the higher latency to enter the open arms and reduced time spent in the open arms compared to their  
193 control littermates (Figure 6b). No changes in the anxiety state level were observed in intact  
194  $ER\alpha^{NesCre}$  males (Figure 6d), castrated and testosterone-supplemented  $ER\alpha^{NesCre}$  mice  
195 (Supplementary Figure 2b), or  $AR::ER\alpha^{NesCre}$  animals (Figures 6f) compared to their corresponding  
196 control littermates.

197 Assessment of aggressive behavior showed that it was completely impaired in both neural  $ER\alpha$  and  
198  $AR::ER\alpha$  knockout males as shown by the very low percentage or absence of mutants showing this  
199 behavior towards non-aggressive intruders compared to their corresponding control littermates  
200 (Figures 6g-h).

201

202 **Discussion**

203 The present study shows that neural *ERα* deletion completely inhibited female sexual  
204 behavior and greatly impaired the gonadotropic axis, confirming the predominant neural role of  
205 *ERα* in female reproduction. In comparison, neural *ERα* deletion impaired the expression of male  
206 sexual behavior and organization of sexually dimorphic neuronal populations in the preoptic area.  
207 Nevertheless, mutant males were able to achieve mating and produce offspring. A greater alteration  
208 of sexual behavior was observed when both neural *ERα* and *AR* were deleted, highlighting the  
209 complementary roles of both receptors in male behavior.

210

211 Neural *ERα* knockout female mice had an arrested estrous cycle, with uterine hypertrophy  
212 and increased estradiol levels and were infertile as evidenced by the absence of corpora lutea. In  
213 mutant females, an altered of pubertal maturation with an advanced time of vaginal opening and  
214 first estrus was also observed (Supplementary Figure 3). These data are in agreement with the  
215 phenotype reported for females lacking *ERα* in neurons [16-17], or in *Kiss1*- [22] and *Tac2*-  
216 expressing cells [21].

217 At the behavioral level, under normalized hormonal levels between the two genotypes, mutant  
218 females were unable to adopt a lordosis posture, despite normal olfactory preference towards males.  
219 The comparable loss of lordosis behavior previously reported for global *ERα* knockout females [30]  
220 can thus be explained at least in part by the altered *ERα* signaling pathway at the neural level.

221 The deficient sexual behavior was probably due to the much lower number of PR-expressing  
222 neurons in the ventromedial hypothalamus, possibly due to the lack of estrogen-induced up-  
223 regulation of PR expression in the absence of neural *ERα*. Indeed, female receptivity during the  
224 estrus stage is triggered by estrogen-induced up-regulation of PR in rodents (for review: [31]).

225 The NesCre transgene used in the present study drives gene excision as early as embryonic day  
226 10.5. It is thus possible that this early *ERα* deletion resulted in a deficient perinatal organization of  
227 brain areas controlling both the expression of sexual behavior and also the gonadotropic axis, thus

228 leading to this infertile phenotype. Indeed, changes in the number of kisspeptin and TH neurons of  
229 the medial preoptic area tend to favor of a lack of feminization of this structure in mutant females.  
230 In comparison, neither a mutation preventing membrane ER $\alpha$  signaling [32] nor neural ER $\alpha$   
231 deletion [14] were found to have any effect on the expression of female sexual behavior or the  
232 organization of related brain structures. Together, these observations support the idea of the  
233 predominant role played by nuclear ER $\alpha$  and its related genomic pathways in the neural control of  
234 female sexual behavior and gonadal axis function.

235

236 In male mice, neural ER $\alpha$  deletion resulted in increased latencies to initiate and achieve  
237 sexual behavior. In particular, sexual experience ameliorated the latency to mount and intromit but  
238 not the time needed to reach ejaculation. This moderate deficiency along with normal olfactory  
239 preference and slightly impaired emission of ultrasonic vocalizations could explain the fertile  
240 phenotype of mutant males. Indeed, neural ER $\alpha$  mutants were able to produce offspring like their  
241 control littermates. These results contrast with the previously reported phenotype of global ER $\alpha$   
242 knockout males, which were infertile and exhibited greatly reduced sexual behavior [33-35]. It is  
243 possible that these huge changes in global knockout males were due to cumulative effects of ER $\alpha$   
244 deletion in neural and peripheral sites; ER $\alpha$  plays a key function in the male urogenital tract.

245 Sex steroid hormones permanently organize the sexually dimorphic neuronal populations of the  
246 preoptic nucleus during the perinatal period. The lower number of calbindin-immunoreactive  
247 neurons and higher number of TH-immunoreactive neurons in neural ER $\alpha$  knockout males are in  
248 favor of an altered perinatal masculinization/defeminization processes in mutant males. It is  
249 possible that the consequences of altered brain organization and resulting sexual dysfunctions were  
250 partly compensated by the increased levels of testosterone, due to the altered negative feedback  
251 exerted by testosterone and its neural estradiol metabolite and unchanged number of AR-  
252 immunoreactive neurons in the neural circuitry controlling sexual behavior. When testosterone

253 levels were normalized between the two genotypes,  $ER\alpha^{NesCre}$  males still exhibited an altered sexual  
254 behavior but the behavioral differences as compared with controls were reduced.

255 We have previously reported a greater impairment of sexual behavior in males lacking the  
256 neural *AR* as evidenced by the observed higher latencies to initiate and achieve sexual behavior and  
257 a more important reduction in the emission of ultrasonic vocalizations together with an hypofertile  
258 phenotype [8, 10, 11, 36]. In these mutant males, no effect of neural *AR* deletion was observed on  
259 sexually dimorphic populations of the preoptic area [10, 37] but changes in cell number, soma size  
260 or dendritic arborization were observed in spinal nuclei involved in erection and ejaculation [11, 12,  
261 37]. Together, these observations support complementary roles of *AR* and  $ER\alpha$  in the control of  
262 sexual behavior in male mice. Accordingly, double knockout males lacking both the *AR* and *ER\alpha* in  
263 the nervous system displayed a more important sexual deficiency than neural *ER\alpha* knockouts.  
264  $AR::ER\alpha^{NesCre}$  males took much longer to initiate mounts and exhibited reduced emission of  
265 ultrasonic vocalizations, suggesting an impaired motivational state. In addition, only half of them  
266 reached ejaculation and those achieving mating took a very long time in comparison with control  
267 littermates with no behavioral improvement after a first sexual experience although olfactory  
268 preference remained unchanged. Altogether, these data strongly suggest that they possibly also  
269 displayed an impaired erectile activity and organization and activation of the spinal nucleus of the  
270 bulbocavernosus as we have previously shown for male mice lacking the neural *AR* [8, 11]. Further  
271 studies will address the reproductive phenotype of  $AR::ER\alpha^{NesCre}$  males in more detail to determine  
272 whether the ejaculating males are able to produce litters in continuous fertility tests.

273 In chemosensory areas located downstream of the olfactory bulb, study is still required of whether  
274 *AR* and  $ER\alpha$  operate in identical or different neuronal populations. In this context, a previous study  
275 showed that *ER\alpha* deletion in inhibitory *Vgat* neurons impaired mating [23]. In addition, a mutation  
276 preventing membrane  $ER\alpha$  signaling triggered altered behavior and reduced number of calbindin-  
277 and kisspeptin-ir neurons [32], thus suggesting the involvement of different  $ER\alpha$  signaling  
278 pathways in these processes.

279

280 Neural *ERα* deletion had no effect on locomotor activity in females. In contrast, it increased  
281 their anxiety-related behavior compared to their control littermates. This may participate to their  
282 inhibited sexual behavior. Comparison with the previous effects for neural *ERβ* on mood behavior  
283 [13-14] suggests that estradiol acts through both *ERα* and *ERβ* signaling pathways to modulate this  
284 behavior.

285 In males, assessment of the effects of neural *ERα* or *AR::ERα* deletion on locomotor activity  
286 showed genotype effects only on the activity of *AR::ERα<sup>NesCre</sup>* males, which was elevated maybe  
287 due to the higher sex steroid levels of these mutants compared to their control littermates. Indeed,  
288 mutant *AR<sup>NesCre</sup>* male mice displayed also a high activity that was restored to control levels by  
289 gonadectomy and supplementation with equivalent concentration of testosterone [8]. No effects of  
290 neural *ERα* or *AR::ERα* deletion were observed on anxiety-related behavior. Estrogens may thus  
291 regulate differently the basal anxiety state level between males and females. In males, if estrogens  
292 seem to be less involved in the modulation of mood behavior, they regulate together with androgens  
293 the expression of social behavior such as aggression. Indeed, suppression of one of the three neural  
294 estrogen or androgen pathways (*ERα*, *ERβ* or *AR*) has been found to be sufficient to greatly impair  
295 or completely inhibit the expression of aggressive behavior ([7, 8, 13], present study).

296

297 In summary, the present data show that neural *ERα* deletion triggers sex differential effects on  
298 reproductive behavior. In females, neural *ERα* is mandatory for the organization and activation of  
299 structures underlying sexual behavior. In males, although *ERα* plays a role in the early  
300 neuroanatomical organization of brain areas involved in sexual behavior, both *AR* and *ERα* are  
301 required for an efficient behavior. These observations were confirmed by the sexual phenotype of  
302 double knockout males lacking both receptors in the nervous system.

303 Neural *ER $\alpha$*  deletion was also found to affect anxiety-related behavior in females but not in males  
304 under non-stressful basal conditions. The data suggest a participation of neural *ER $\alpha$*  signaling  
305 pathway, as for neural *ER $\beta$*  [13-14], in estrogen-dependent modulation of anxiety-related behavior.  
306 In males, while the neural contribution of *ER $\alpha$*  pathway seems less important in the regulation of  
307 anxiety-related behavior, both pathways together with *ER $\beta$*  [13] are important for the expression of  
308 aggressive behavior. It would be interesting for further studies to identify the neuronal versus glial  
309 contribution of these signaling pathways across life stages.

310

311 **Methods**

312 **Animals.**

313 The  $ER\alpha^{NesCre}$  mouse line was obtained, on a mixed C57BL/6 x CD1 background, by crossing  
314 floxed  $ER\alpha$  females in which the exon 3 of  $ER\alpha$  was flanked by loxP sites [38] with floxed  $ER\alpha$   
315 males expressing the Cre recombinase under the control of the rat Nestin promoter (NesCre  
316 transgene; [39]). Mutant mice ( $ER\alpha^{fl/fl}$ , NesCre:  $ER\alpha^{NesCre}$ ) and their control littermates ( $ER\alpha^{fl/fl}$ )  
317 were used. To obtain the double  $AR::ER\alpha^{NesCre}$  mouse line, we first generated animals carrying  
318 floxed  $ER\alpha$  and  $AR$  genes by crossing  $ER\alpha^{fl/fl}$  and  $AR^{fl/fl}$  mouse lines. Homozygous floxed females  
319 ( $ER\alpha^{fl/fl}::AR^{fl/fl}$ ) were then crossed with  $ER\alpha^{NesCre}$  males to generate mutant males ( $ER\alpha^{fl/fl}::AR^{fl/Y}$ ,  
320 NesCre:  $AR::ER\alpha^{NesCre}$ ) and their control littermates ( $ER\alpha^{fl/fl}::AR^{fl/Y}$ ;  $AR::ER\alpha^{fl/fl}$ ). After weaning,  
321 mice were kept at 22°C under an inverted light schedule, i.e. the dark time began at 1:30 PM with a  
322 12:12 hour light-dark cycle and fed a standard diet with free access to food and water.  
323 All experiments were conducted on adult animals of 3 to 6 months of age, in compliance with the  
324 French and European legal requirements (Decree 2010/63/UE) and were approved by the “Charles  
325 Darwin” Ethical committee (project number 01490-01).

326

327 **PCR and quantitative RT-PCR.**

328 The selective neural  $ER\alpha$  deletion was assessed by PCR and qRT-PCR as previously described [8,  
329 38]. For PCR, control and mutant females were sacrificed and brain and ovaries were collected.  
330 Detection of the Cre transgene and the floxed or truncated  $ER\alpha$  allele was carried out using the  
331 following primers:  $ER\alpha$  forward: 5'-AGG-CTT-TGT-CTC-GCT-TTC-C-3';  $ER\alpha$  reverse: 5'-GAT-  
332 CAT-TCA-GAG-AGA-CAA-GAG-GAA-CC-3'; NesCre forward: 5'-GCC-TGC-ATT-ACC-GGT-  
333 CGA-TGC-AAC-GA-3'; reverse: 5'- GTG-GCA-GAT-GGC-GCG-GCA-ACA-CCA-TT-3'.  
334 Amplification conditions were: 5 min at 95°C for denaturation, 40 cycles comprising 30 s at 95°C for  
335 denaturation, 30 s at 58°C for annealing and 1 min at 72°C for elongation and then 10 min at 72°C  
336 for final elongation.



337 For semi-quantitative RT-PCR, ovaries, testes and three brains areas (cortex, hippocampus,  
338 hypothalamus) were collected and rapidly frozen with dry ice. Total RNAs were extracted using  
339 TRIzol reagent (Invitrogen, Carlsbad, USA) and 1  $\mu$ g was reverse transcribed into cDNA using the  
340 Superscript III First-Strand Synthesis System (Invitrogen Carlsbad, USA) and random hexamers.  
341 PCR reactions were performed using the resulting cDNA (4  $\mu$ l), dNTPs (10 nM each), primers (100  
342  $\mu$ M, Eurogentec, Seraing, Belgium) and Taq DNA pol (Invitrogen) in a MyCycler Thermal Cycler  
343 (Bio Rad, Marne la Coquette, France). Primers for ER $\alpha$  (forward: 5'-CGT-GTG-CAA-TGA-CTA-  
344 TGC-CTC-T-3'; reverse: 5'- TGG-TGC-ATT-GGT-TTG-TAG-CTG-G -3') and GADPH (forward:  
345 5'- TGC-ACC-ACC-AAC-TGC-TTA-GC -3'; reverse: 5'- GGC-ATG-GAC-TGT-GGT-CAT-GAG  
346 -3') were used. Amplification conditions were: 5 min at 95°C for denaturation, 40 cycles comprising  
347 30 s at 95°C for denaturation, 30 s at 56°C for annealing and 1 min at 72°C for elongation and then  
348 10 min at 72°C for final elongation. The amplified cDNA fragments were separated by  
349 electrophoresis in a 1.5% agarose gel and stained by ClearSight DNA stain (Euromedex-BM,  
350 Souffelweyersheim).

351

### 352 **Reproductive physiology.**

353 Prepubertal females were monitored daily for visual evidence of vaginal opening from postnatal day  
354 15 and vaginal smears flushed with physiological saline were taken daily and colored with  
355 hematoxylin-eosin to identify the estrus stage based on cell types [14, 40]. Adult females were also  
356 examined daily for the estrous cycle for 3 weeks using the same protocol, and were sacrificed at the  
357 diestrus phase to collect trunk blood and weigh ovaries and uteri. Ovaries were fixed in Bouin's  
358 buffer, washed in ethanol, paraffin-embedded and sliced into 5  $\mu$ m sections [40]. The sections were  
359 mounted and deparaffinized, stained with hematoxylin–eosin and the number of corpus lutea was  
360 analyzed.

361 At sacrifice of males, blood was collected and body, testis and seminal vesicles were weighed. The  
362 epididymis was dissected and sperm collected, incubated in PBS for 10 minutes at 37°C, and

363 centrifuged 5 minutes at 100 x g. The supernatant was removed and 10-fold diluted [8] and  
364 spermatozoa were counted in a hemocytometer with a Normaski microscope. Fertility was assessed  
365 in continuous mating for 5 months. Each male was paired with two age-matched control females. The  
366 interval from mating to the first litter and the number and size of litters were reported.  
367 Sera obtained by decantation and centrifugation (2500 x g for 15 minutes) were used to measure by  
368 radioimmunoassay circulating levels of estradiol using DSL-4800 kit (Beckman-Coulter), and  
369 testosterone by the platform of the laboratory of Behavioral and Reproductive Physiology (UMR  
370 0085 INRAE-CNRS- IFCE-University of Tours).

371

### 372 **Experimental conditions for behavioral tests.**

373 Assays were conducted under red-light illumination 2 hours after lights-off and were videotaped for  
374 later analyses. The devices used in the tests, with the exception of animal home cages, were cleaned  
375 with 10% ethanol between trials. The analyses were performed by blind observation.

376 Stimulus C57BL/6J females used for the analyses of male behaviors and control ( $ER\alpha^{fl/fl}$ ) and mutant  
377 ( $ER\alpha^{NesCre}$ ) females were ovariectomized under general anesthesia (xylazine 10 mg/kg, ketamine 100  
378 mg/kg), and implanted with 1 cm sc SILASTIC implants (3.18 mm outer diameter X 1.98 mm inner  
379 diameter; Dow Corning, Midland, MI) filled with 50  $\mu$ g of estradiol-benzoate (Sigma-Aldrich)  
380 diluted in 30  $\mu$ l of sesame oil. Females were subcutaneously injected with 1 mg of progesterone  
381 (Sigma-Aldrich) in 100  $\mu$ l of sesame oil 4 to 5 hours before the tests to experimentally induce their  
382 receptivity. The stimulus C57BL/6J females used for the analyses of male behaviors were all  
383 sexually experienced twice and screened before each mating test for their lordosis behavior with stud  
384 males.

385 Control ( $ER\alpha^{fl/fl}$ ) and mutant ( $ER\alpha^{NesCre}$ ) males were gonadectomized under similar experimental  
386 conditions and implanted with a subcutaneous SILASTIC tubes containing 10 mg of testosterone  
387 (Sigma–Aldrich). Stimulus C57BL/6J males used for the analyses of female behaviors were sexually

388 experienced and selected for showing a strong mounting behavior in less than 5 minutes after the  
389 presentation of a female (Supplementary Figure 4).

390

391 **Sexual behavior.**

392 Sexually naive females were tested for their sexual receptivity toward male mounting as previously  
393 described [14]. Test began when the female was introduced into the home cage of a sexually  
394 experienced C57BL/6J stud male and lasted for 20 minutes or until the female received 20 mounts.  
395 Females were tested three times with an interval of 1 week between tests. The lordosis quotient  
396 (number of lordosis responses/number of mounts) was reported.

397 Sexually naive males were housed in individual cages 4 days before the beginning of the tests. Each  
398 male was tested in its home cage for 10 hours after the introduction of a sexually experienced  
399 stimulus female. Males were tested twice in tests 1 (naive) and 2 (sexually-experienced) with a time  
400 interval of one week. Male sexual behavior was analyzed by scoring the latency to initiate mounts,  
401 intromissions and to reach ejaculation, and the frequency of mounts, intromissions, thrusts as  
402 previously described [8]. Mating duration was defined as the time from the first mount to ejaculation  
403 and the ratio intromission as the proportion of mounts with successful intromission. A latency of 600  
404 minutes was given to males that did not initiate the behavior.

405

406 **Olfactory preference.**

407 Sexually experienced males and females were tested for odor preference in a Y-maze apparatus as  
408 previously described [10, 14, 41]. Female mice were allowed to become familiar with the maze for  
409 10 minutes over two consecutive days. On the experimental test day, each Y-arm randomly  
410 received either an anesthetized stimulus male or female. Subjects were free to investigate the  
411 stimuli for 9 min. Time spent in investigation by the tested animals was recorded and expressed as a  
412 percentage of the total time of investigation.

413

414 **Ultrasonic vocalizations.**

415 Each sexually-experienced male was tested in its home cage in the presence of a receptive female.  
416 The vocalizations were recorded for 4 minutes, after the introduction of a female, with an  
417 Ultrasound gate microphone CM16/CMPA (Avisoft Bioacoustics), connected to an Ultrasound  
418 recording interface plugged into a computer equipped with the Avisoft-SASLab Pro 5.2.09  
419 recording software. The recordings were analyzed using SASLab Pro (Avisoft Bioacoustics). A  
420 cutoff frequency of 30 kHz was used and elements were separated based on an automatic threshold  
421 with a hold time of 15 ms. [36, 42]. The total number and duration of total ultrasonic vocalizations  
422 and syllables were reported.

423

424 **Locomotor activity.**

425 Activity was analyzed in a computed circular corridor made up of two concentric cylinders and  
426 crossed by four diametrically opposite infrared beams (Imetronics) as previously described [8, 14].  
427 The locomotor activity was counted when animals interrupted two successive beams and had thus  
428 traveled a quarter of the circular corridor. The cumulative activity was reported.

429

430 **Anxiety-related behavior.**

431 The anxiety state was assessed in the elevated O-maze paradigm as previously described [14]. The  
432 test lasted 9 min and began when the animal was placed in a closed arm. The latency to enter into  
433 an open arm (with the four paws out) and the time spent in the open arms were analyzed.

434

435 **Aggressive behavior.**

436 Each sexually experienced male was tested in the resident-intruder test, after one week without  
437 bedding change, as previously described [7-8]. Tests lasted 10 minutes and began when a sexually  
438 inexperienced gonadally intact adult A/J male (The Jackson Laboratory, USA) was introduced into  
439 the home-cage of the tested resident male. Each resident male was exposed to a different intruder on

440 three consecutive days. Aggressive behavior was defined as lunging, biting, wrestling and kicking.  
441 The proportion of males showing aggressive behavior was reported.

442

#### 443 **Tissu preparation.**

444 Brains from perfused animals were removed, postfixed overnight in 4% PFA, cryoprotected in 0.1  
445 M phosphate buffer containing 30% sucrose and stored at  $-80^{\circ}\text{C}$ . Coronal sections ( $30\ \mu\text{m}$ ) were  
446 then processed for immunolabeling as previously described [10, 14].

447

#### 448 **AR, TH and Calbindin immunostainings.**

449 Endogenous peroxidases were blocked for 30 minutes in PBS containing 3%  $\text{H}_2\text{O}_2$  and sections  
450 were incubated in blocking solution (PBS containing 0.2% Triton X-100 and 1% bovine serum  
451 albumin for AR or 5% horse serum for TH and Calbindin) 2 h at room temperature (RT). Sections  
452 were incubated at  $4^{\circ}\text{C}$  in the blocking solution supplemented with 1:200 diluted rabbit anti-AR (sc-  
453 816, Santa Cruz Biotechnology) for 36 hours, or with 1:2000 diluted mouse anti-TH antibody  
454 (MAB318, Millipore) or 1:1000 diluted mouse anti-Calbindin antibody (C9848, Sigma-Aldrich)  
455 overnight. Sections were incubated in PBS-0.2%Triton solution supplemented with 1:1000 diluted  
456 biotinylated secondary antibody (goat anti-rabbit BA1000, Vector; or horse anti-mouse BA2000,  
457 Vector) for 2 hours at RT. Signals were amplified with VECTASTAIN<sup>®</sup> ABC kit (Vector) 1:1000  
458 diluted in PBS for 1 hour at RT and visualized with 3,3'-diaminobenzidine tetrahydrochloride  
459 (DAB 0.02%, 0.01%  $\text{H}_2\text{O}_2$  in 0.05 M Tris, pH 7.4). Sections were mounted on slides, dehydrated  
460 and coversliped using Permount (Sigma-Aldrich). The slices were scanned using an automatic slice  
461 scanner (NanoZoomer, Hamamatsu). Regions of interest were drawn based on the Mouse Brain  
462 Atlas [43] and immunoreactive cells were manually counted using the NDPview software. AR  
463 positive nuclei quantification was performed bilaterally on one representative section of each area  
464 (MeA: plate 43; BNST-MPOA: plate 30; LS: plate 27). TH positive cells quantification was  
465 performed bilaterally on five sections covering the entire AVPV/PeN (plates 28 to 33).

466 Quantification of calbindin-positive cells was performed on one representative section of the SDN-  
467 POA (plate 33).

468

#### 469 **Kisspeptin and PR labelings.**

470 Sections were incubated in a blocking solution (PBS containing 0.3% Triton X-100 and 3% donkey  
471 serum) 2 hours at RT and then at 4°C for 36 hours in the blocking solution supplemented with 1:10  
472 000 diluted sheep anti-kisspeptin (AC053, generous gift of I. Franceschini [44]) or 1:100 rabbit  
473 anti-PR (A0098, Dako) antibodies. The sections were incubated in PBS-0.3%Triton solution  
474 supplemented with 1:500 donkey anti-sheep-Alexa 488 (A11015, Life Technologies) or 1:1000  
475 biotinylated horse anti-rabbit (BA1100, Vector) secondary antibodies for 2 hours at RT. The signal  
476 was amplified with a Cy3-Streptavidin (s32355, Life Technologies) 1:1000 diluted in PBS for 1  
477 hour at RT. The sections were counterstained in a 2 µg/ml Hoechst solution (Invitrogen) for 5 min,  
478 mounted on slides and coverslipped with Mowiol. Three sections covering the AVPV/PeN (plates  
479 28-31 of the Paxinos and Franklin Mouse Brain Atlas) were imaged using an inverting microscope  
480 equipped with an apotome (Axiovert 200M coupled to the AxioCam MRm camera, Zeiss). Z-stacks  
481 (3 µm) were carried out on the entire thickness of the sections. Kisspeptin positive cells were  
482 manually counted on using the ImageJ software (NIH). PR positive nuclei quantification was  
483 performed bilaterally on one representative section of the VMHvl (plates 45 of the Paxinos and  
484 Franklin Mouse Brain Atlas).

485

#### 486 **Statistics and Reproducibility.**

487 Analyses were performed using GraphPad Prism 8.0 (GraphPad Software, Inc.) Assumption of  
488 normality (Shapiro-Wilk test) was tested before conducting the following statistical analyses. Two-  
489 way repeated measures ANOVA was used to analyze the effect of genotype and stimulus or  
490 genotype and time on olfactory preference, lordosis quotient for ER $\alpha$ <sup>NesCre</sup> males and females and  
491 AR::ER $\alpha$ <sup>NesCre</sup> males. One-way ANOVA with Tukey's post hoc was used to analyze group effect on

492 kisspeptin-immunoreactivity for  $ER\alpha^{NesCre}$  females. Mann-Whitney test was used to compare estrous  
493 cycle duration, the latency, number of entries and time spent in the O-maze open arm for  $ER\alpha^{NesCre}$   
494 females. It was also used to compare in males the latencies to mount and intromit, the number of  
495 mounts and pelvic thrusts in sexually naive and experienced  $ER\alpha^{NesCre}$  males; the number of pelvic  
496 thrusts in sexually naive  $ER\alpha^{NesCre}$  males and the interval between intromissions in sexually  
497 experienced  $ER\alpha^{NesCre}$  males, the number of short, downward, modulated and one-jump USV  
498 syllables, the duration of short, upward, downward, modulated, mixed and one-jump USV syllables,  
499 the levels of plasma testosterone and estradiol, the number of spermatozoa, the body weight and  
500 relative testis weight in  $ER\alpha^{NesCre}$  males, as well as the latencies to mount, intromit and ejaculate,  
501 the mating duration and the interval between intromissions in sexually naive and experienced  
502  $AR::ER\alpha^{NesCre}$  males, the latency to enter, the number of entries and the time spent in the O-maze  
503 open arm, the body weight and the relative testis weight in  $AR::ER\alpha^{NesCre}$  males. The remaining data  
504 were analyzed by a two-tailed unpaired Student t-test. The probability of error level (alpha) was  
505 chosen to be 0.05 (\*  $< 0.05$ , \*\* $p < 0.01$  and \*\*\* $p < 0.001$ ). Results are presented as means  $\pm$  SEM,  
506 except for the percentage of ejaculating  $ER\alpha^{NesCre}$  and  $AR::ER\alpha^{NesCre}$  males and the percentage of  
507 aggressive  $ER\alpha^{NesCre}$  and  $AR::ER\alpha^{NesCre}$  males, which were analyzed by Fisher's exact test.  
508

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513

514 **Author contributions**

515 SMK and ACT designed the study. SR provided the floxed ER $\alpha$  mouse line. ACT, SD, LN, DC and  
516 CP carried out the experiments. ACT, SD, HHP and SMK analyzed the data. SMK and ACT wrote  
517 the manuscript and all authors read and approved the final manuscript.

518

519 **Competing interests**

520 The authors declare that they have no competing interests.

521

522 **Data Availability statement**

523 Source data is provided as Supplementary Data 1.

524



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- 658

659 **Figures captions**

660 **Figure 1. Selective neural *ERα* deletion.** **a.** Representative PCR analyses performed on ovaries  
661 and brains from control ( $ER\alpha^{fl/fl}$ ) and mutant females ( $ER\alpha^{NesCre}$ ). The upper panels show the  
662 presence of the floxed *ERα* allele (881 bp) in the ovaries of both genotypes and in the brain of  
663 controls, and the deleted allele (223 bp) in the brain of mutant females expressing the Cre-  
664 recombinase in the lower panels. DNA size markers at 100 bp increments are shown in the left  
665 column of the four panels. **b-c.** Levels of *ERα* mRNA normalized to *Gapdh* levels in the ovary,  
666 cortex, hippocampus (HPC) and hypothalamus (HPT) of females (**b**), or in the testis, cortex, HPC  
667 and HPT of males (**c**). Data are means  $\pm$  S.E.M. of 3 mice per genotype (\* $p < 0.05$ , \*\* $p < 0.01$   
668 versus controls).

669  
670 **Figure 2. Neural *ERα* deletion induced severe deficit of female reproductive function and**  
671 **behavior.** **a.** Cumulative days in stages of the estrous cycle for control ( $ER\alpha^{fl/fl}$ ) and mutant females  
672 ( $ER\alpha^{NesCre}$ ). PE: proestrus, Es: estrus, ME: metestrus, DE: diestrus. Data are means  $\pm$  S.E.M for 28-  
673 35 females per genotype; \*\*\* $p < 0.001$  versus controls. **b-c.** Representative images of kisspeptin-  
674 immunoreactivity in the anteroventral periventricular area (AVPV) of control females at PE-Es or  
675 ME-DE and of mutant littermates (**b**). Kisspeptin positive neurons are highlighted by yellow arrow  
676 heads (scale bars = 25  $\mu$ m), and quantitative data for kisspeptin-ir cell number (**c**) in 5-6 females per  
677 group. General effect analyzed by ANOVA (### $p < 0.001$  or # $p < 0.05$ ) and post hoc analyses (\* $p$   
678  $< 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ) versus the indicated group are represented. **d-e.** Representative  
679 images of tyrosine hydroxylase (TH)-immunoreactivity (ir) in the AVPV of control and mutant  
680 females (scale bars = 50  $\mu$ m) (**d**), and quantitative data for TH-ir cell number (**e**) in 6-11 females  
681 per genotype. \* $p < 0.05$  versus the control group. **f.** Lordosis quotient measured in ovariectomized  
682 and hormonally-primed females (n = 11-13 per genotype) over three tests with a week interval  
683 between two consecutive tests. Genotype effect (### $p < 0.001$ ) and posthoc analyses (\* $p < 0.05$ ,  
684 \*\*\* $p < 0.001$ ) versus controls are indicated. **g.** Olfactory preference of sexually experienced

685 females (n = 11-13 per genotype), expressed as percentage of total time spent sniffing male or  
686 female stimuli in a Y-maze. The stimulus effect analyzed by two-way ANOVA (###p < 0.001) is  
687 indicated. **h-i**. Quantitative data (**h**) and representative image of progesterone receptor (PR)-ir in the  
688 ventromedial hypothalamus (VMH) (**i**), n = 6 per genotype, \*\*\*p < 0.001 versus controls. Scale  
689 bars = 50  $\mu$ m. All data are shown as means  $\pm$  S.E.M with individual values.

690

691 **Figure 3. The effects of neural ER $\alpha$ <sup>NesCre</sup> deletion on sexual behavior in males. a.** Percentage of  
692 control (ER $\alpha$ <sup>fl/fl</sup>) and mutant males (ER $\alpha$ <sup>NesCre</sup>) reaching ejaculation in tests 1 (naive animals) and 2  
693 (sexually experienced). Data are shown as percentages. **b.** Latencies to mount (M), intromit (MI)  
694 and to reach ejaculation (Ej) for control and mutant males (n = 10 per genotype) on Test 1 and Test  
695 2. Significant differences between the two genotype are indicated; \*p < 0.05, \*\*p < 0.01, \*\*\*p <  
696 0.001 versus controls. **c.** Mating duration from the first mount to ejaculation was higher in mutant  
697 males (\*\*\*p < 0.001 and \*\*p < 0.01 in Tests 1 and 2, respectively) (n = 10 per genotype) **d.**  
698 Olfactory preference of sexually experienced control and mutant male mice (n = 11 per genotype),  
699 expressed as the percentage of total time spent sniffing the stimuli. The stimulus effect (#p < 0.01)  
700 is indicated. **e.** Representative sound waveform for control and mutant males in the presence of a  
701 sexually receptive female during a 4-min test. **f-g** Total number of emitted ultrasonic vocalizations  
702 USV (**f**) and number of each syllable (**g**) produced by sexually experienced males (n = 10-11 per  
703 genotype). **h-i** Total duration of emitted USV (**h**) and duration of each syllable (**i**). \*p < 0.05, or \*\*p  
704 < 0.01 versus controls (n = 10-11 per genotype). Instead stated otherwise, data are shown as means  
705  $\pm$  S.E.M with individual values.

706

707 **Figure 4. Neural ER $\alpha$  deletion altered the organization of sexually dimorphic populations. a.**  
708 Representative calbindin-immunoreactivity (ir) in the medial preoptic area (MPOA) of control  
709 (ER $\alpha$ <sup>fl/fl</sup>; *left*) and mutant males (ER $\alpha$ <sup>NesCre</sup>; *right*). Scale bar 100  $\mu$ m. **b.** Quantification of calbindin-  
710 ir neurons in control and mutant males (n = 7-8 per genotype), \*\*\*p < 0.001 vs controls. **c.**



711 Representative images of tyrosine hydroxylase (TH)-ir in the in the anteroventral periventricular  
712 area (AVPV) of control (*left*) and mutant (*right*) males. Scale bar 50  $\mu$ m. **d.** Quantification of TH-  
713 positive neurons in the AVPV, \* $p < 0.05$  versus controls (n = 7-8 per genotype). **e.** Representative  
714 androgen receptor (AR)-ir in the medial amygdala (MeA; *left*) and the MPOA (*right*). Scale bars =  
715 200  $\mu$ m. **f.** Quantification of the number of AR-ir neurons in the MeA, bed nucleus of stria  
716 terminalis (BNST), MPOA and lateral septum (LS) in controls and mutants (n = 10-15 males per  
717 genotype);  $p = 0.3$  and  $0.9$ , respectively. All data are shown as means  $\pm$  S.E.M with individual  
718 values.

719

720 **Figure 5. The behavioral effects of double  $AR::ER\alpha$  deletion in males.** **a.** Percentage of control  
721 ( $AR::ER\alpha^{fl/fl}$ ) and mutant ( $AR::ER\alpha^{NesCre}$ ) males reaching ejaculation in tests 1 (naive animals) and  
722 2 (sexually-experienced animals). Data are shown as percentages. **b.** Behavioral components of  
723 sexual behavior for control and mutant males (n = 7-11 per genotype). Latencies to mount (M),  
724 intromit (MI) and to reach ejaculation (Ej) in Tests 1 and 2; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$   
725 versus controls. **c.** Mating duration from the first mount until ejaculation;  $p = 0.2$  for Test1 and  
726 \*\*\* $p < 0.001$  for Test 2 (n = 7-11 per genotype). **d.** Olfactory preference of sexually experienced  
727 controls and mutants (n = 7-11 per genotype) expressed as the percentage of total time spent  
728 sniffing stimuli. The effect of stimulus (# $p < 0.05$ ) is indicated. **e.** Total number (left) and duration  
729 (right) of ultrasonic vocalizations (USV) produced by controls and mutants (n = 7-11 per genotype)  
730 in the presence of a sexually receptive female during a 4-min test. \*\* $p < 0.01$  and \* $p < 0.05$  versus  
731 control littermates. Instead stated otherwise, data are shown as means  $\pm$  S.E.M with individual  
732 values.

733

734 **Figure 6. Effect of neural  $ER\alpha$  or  $AR::ER\alpha$  deletion on locomotor activity, anxiety-related and**  
735 **aggressive behavior.** **a, c, e.** Cumulative locomotor activity recorded in a circular corridor during  
736 the 2 h-test for control ( $ER\alpha^{fl/fl}$ ) and mutant ( $ER\alpha^{NesCre}$ ) females (**a**, n = 11-13 per genotype), control

737 (ER $\alpha^{fl/fl}$ ) and mutant (ER $\alpha^{NesCre}$ ) males (**c**, n = 10-11 per genotype), or control (AR::ER $\alpha^{fl/fl}$ ) and  
738 mutant (AR::ER $\alpha^{NesCre}$ ) males (**e**, n = 7-11 per genotype). **b, d, f**. Anxiety-related behavior measured  
739 in the elevated O-maze for control (ER $\alpha^{fl/fl}$ ) and mutant (ER $\alpha^{NesCre}$ ) females (**b**, n = 11-13 per  
740 genotype), control (ER $\alpha^{fl/fl}$ ) and mutant (ER $\alpha^{NesCre}$ ) males (**d**, n = 10-11 per genotype), or control  
741 (AR::ER $\alpha^{fl/fl}$ ) and mutant (AR::ER $\alpha^{NesCre}$ ) males (**f**, n = 7-11 per genotype). The latency to enter the  
742 open arm (*left*) and the time spent in the open arm (*right*) were analyzed. **g-h**. Percentage of males  
743 from the ER $\alpha^{NesCre}$  (**g**) or AR::ER $\alpha^{NesCre}$  (**h**) mouse lines displaying aggressive behavior towards non-  
744 aggressive males in the resident-intruder test across two or three tests. \*p < 0.05, \*\*p < 0.01 versus  
745 the control group. Data are shown as means  $\pm$  S.E.M with individual values (panels a to f) or as  
746 percentage (panels g and h).

747

748 **Table 1. Sexual behavior of gonadally intact ER $\alpha$ <sup>NesCre</sup> males**

| Behavioral events                        | Sexually naive (Test 1)      |                               | Sexually experienced (Test 2) |                               |
|------------------------------------------|------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                                          | ER $\alpha$ <sup>fl/fl</sup> | ER $\alpha$ <sup>NesCre</sup> | ER $\alpha$ <sup>fl/fl</sup>  | ER $\alpha$ <sup>NesCre</sup> |
| Number of Mounts (M)                     | 6.4 ±1.7                     | 15.2 ±3.2 *                   | 5.7 ±1.8                      | 13.3 ±2.8 *                   |
| Number of Mounts with intromissions (MI) | 26.6 ±4.7                    | 52.5 ±7.1 **                  | 25.6 ±4.7                     | 45.2 ±7.1 *                   |
| Intromission ratio MI/M+MI (%)           | 78 ±5                        | 76 ±4                         | 82 ±5                         | 78 ±3                         |
| MI duration (sec)                        | 17.9 ±2.1                    | 17.6 ±1.2                     | 18.6 ±1.7                     | 19.6 ±1.5                     |
| MI interval (sec)                        | 43.7 ±7.9                    | 80.9 ±7.7 **                  | 22.8 ±2.7                     | 83.4 ±26.1 ***                |
| Pelvic thrusts                           | 531.2 ±83.2                  | 1026.2 ±150.5 *               | 560.3 ±100.6                  | 995.1 ±129.8 *                |

749

750 Quantification of sexual behavior components displayed by intact sexually naive and experienced  
 751 control (ER $\alpha$ <sup>fl/fl</sup>) and mutant (ER $\alpha$ <sup>NesCre</sup>) males. Data are means ± S.E.M. for 10 males per genotype.

752 \*p < 0.05, \*\*\*p < 0.01; \*\*\*\*p < 0.001 versus control littermates.

753

754 **Table 2. Sexual behavior of castrated and testosterone supplemented ER $\alpha$ <sup>NesCre</sup> males**

|                                | ER $\alpha$ <sup>fl/fl</sup> | ER $\alpha$ <sup>NesCre</sup> |
|--------------------------------|------------------------------|-------------------------------|
| Seminal vesicle weight (%bw)   | 1.44 ± 0.12                  | 1.58 ± 0.11                   |
| Latency to behavior (min)      |                              |                               |
| Mounts (M)                     | 2.32 ± 0.26                  | 16.73 ± 6.70 *                |
| Mounts with intromissions (MI) | 2.85 ± 0.27                  | 17.40 ± 7.22 *                |
| Ejaculation                    | 46.26 ± 12.34                | 160.23 ± 44 *                 |
| Mating duration (min)          | 43.8 ± 12.35                 | 143.50 ± 47.05 *              |
| Number of events               |                              |                               |
| Mounts (M)                     | 8.63 ± 2.79                  | 6.86 ± 2.72                   |
| Mounts with intromissions (MI) | 15.75 ± 2.91                 | 12.71 ± 4.38                  |
| Pelvic thrusts                 | 672.75 ± 107.72              | 603.43 ± 148.24               |
| Intromission ratio MI/M+MI (%) | 68.01 ± 8.18                 | 67.26 ± 3.68                  |

755

756 The mean weight ± S.E.M of seminal vesicles is indicated as percentage of body weight (bw) for  
 757 control (ER $\alpha$ <sup>fl/fl</sup>) and mutant (ER $\alpha$ <sup>NesCre</sup>) males, which were gonadectomized and normalized for  
 758 their testosterone levels (9-10 males per genotype). The latencies and behavioral events are  
 759 indicated for sexually experienced males that reached ejaculation (7-8 males per genotype). Data  
 760 are means ± S.E.M. \*p < 0.05 versus control littermates.

761

762 **Table 3. Sexual behavior of AR::ER $\alpha$ <sup>NesCre</sup> males.**

| Behavioral events                        | Sexually naive (Test 1)          |                                   | Sexually experienced (Test 2)    |                                   |
|------------------------------------------|----------------------------------|-----------------------------------|----------------------------------|-----------------------------------|
|                                          | AR::ER $\alpha$ <sup>fl/fl</sup> | AR::ER $\alpha$ <sup>NesCre</sup> | AR::ER $\alpha$ <sup>fl/fl</sup> | AR::ER $\alpha$ <sup>NesCre</sup> |
| Number of Mounts (M)                     | 8.27 ± 1.90                      | 8.57 ± 2.57                       | 6.72 ± 1.57                      | 30.85 ± 9.18 **                   |
| Number of Mounts with intromissions (MI) | 21.0 ± 1.99                      | 10.28 ± 3.44 *                    | 14.18 ± 2.33                     | 23.71 ± 5.42                      |
| Intromission ratio MI/M+MI (%)           | 72.51 ± 4.23                     | 44.9 ± 9.04 **                    | 69.19 ± 4.69                     | 46.28 ± 7.21 *                    |
| MI duration (sec)                        | 24.71 ± 2.35                     | 19.19 ± 4.70                      | 28.51 ± 3.61                     | 22.03 ± 5.77                      |
| MI interval (sec)                        | 37.82 ± 5.75                     | 6125.97 ± 5012.20                 | 24.20 ± 3.24                     | 387.52 ± 131.23 **                |
| Pelvic thrusts                           | 570.63 ± 49.05                   | 153.57 ± 49.65 ***                | 443.32 ± 46.65                   | 357.14 ± 98.74                    |

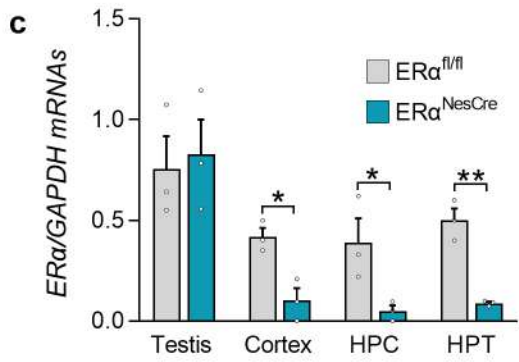
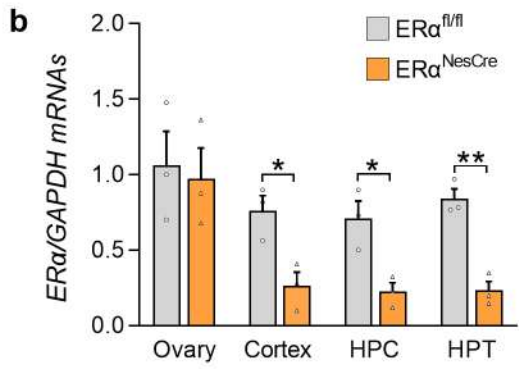
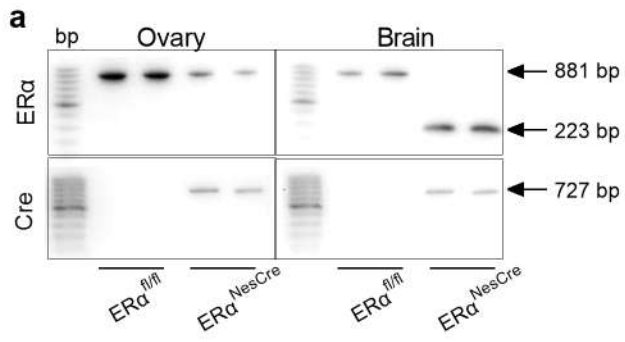
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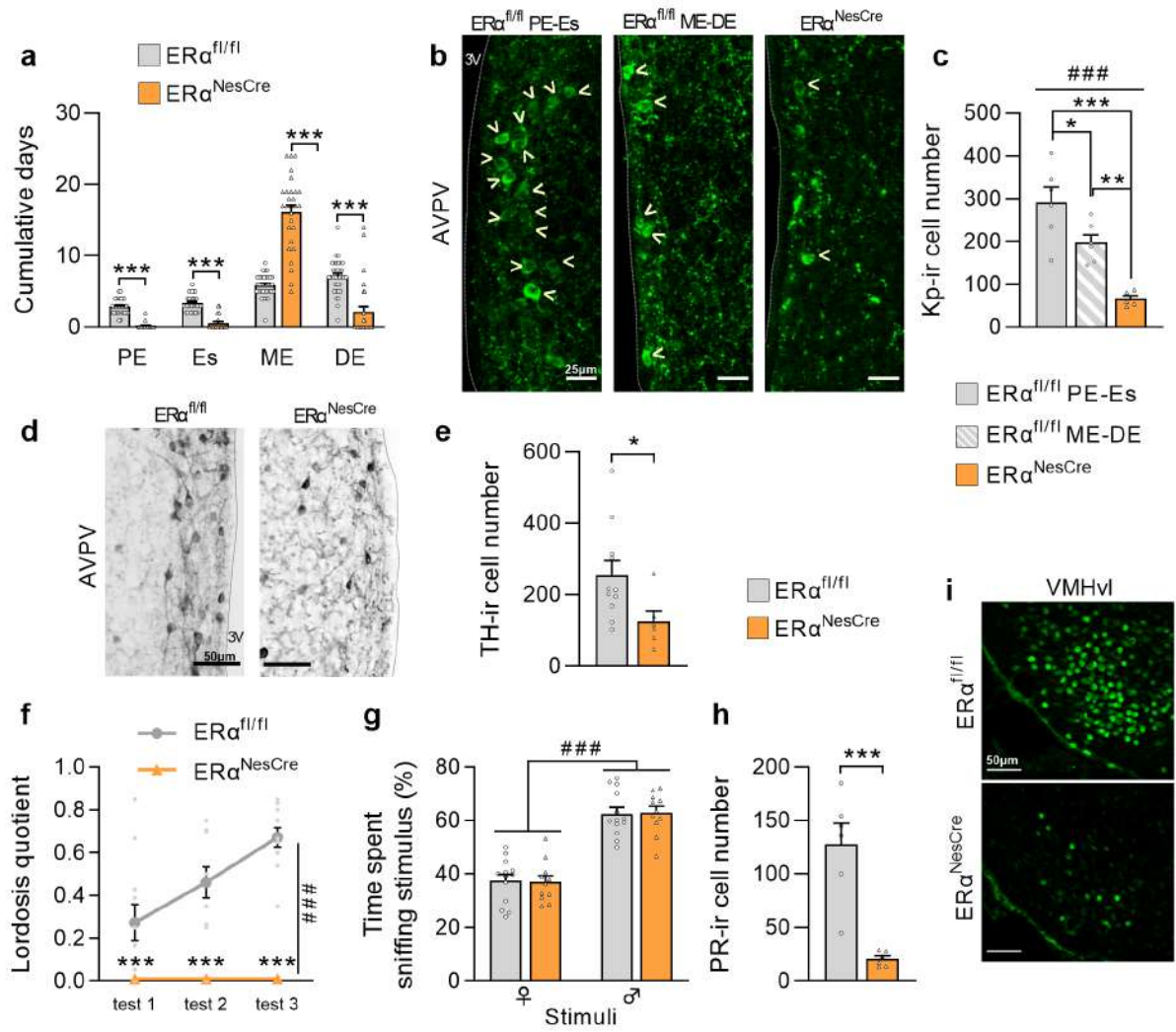
764 Quantification of sexual behavior components displayed by sexually naive and experienced control

765 AR::ER $\alpha$ <sup>fl/fl</sup> and mutant AR::ER $\alpha$ <sup>NesCre</sup> males. Data are means ± S.E.M. for 7 to 11 males per

766 genotype. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 versus control littermates.

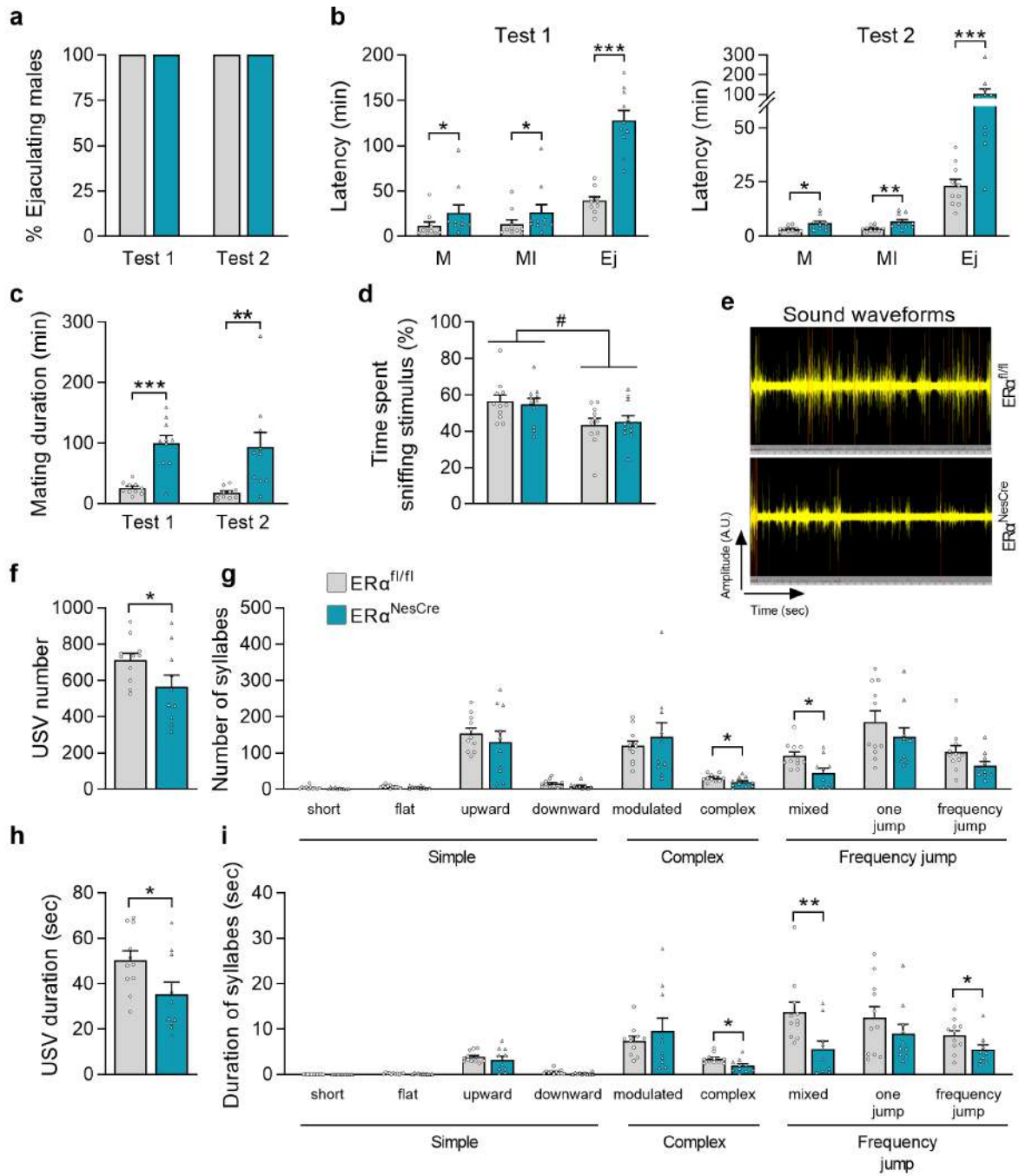
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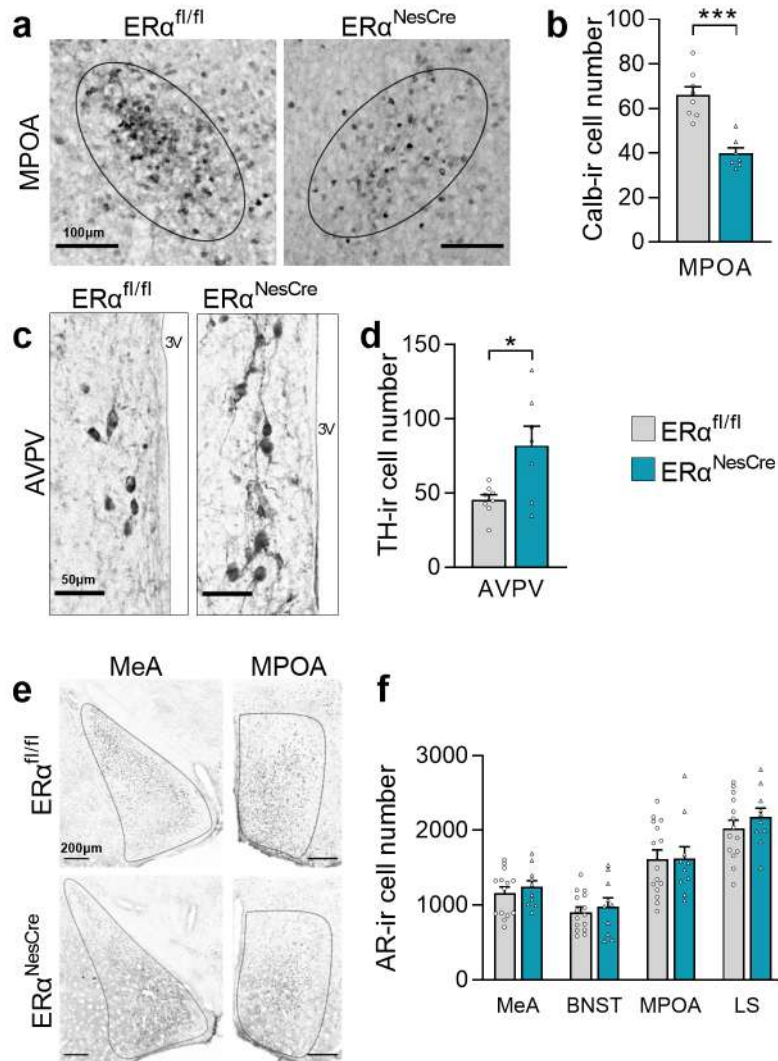
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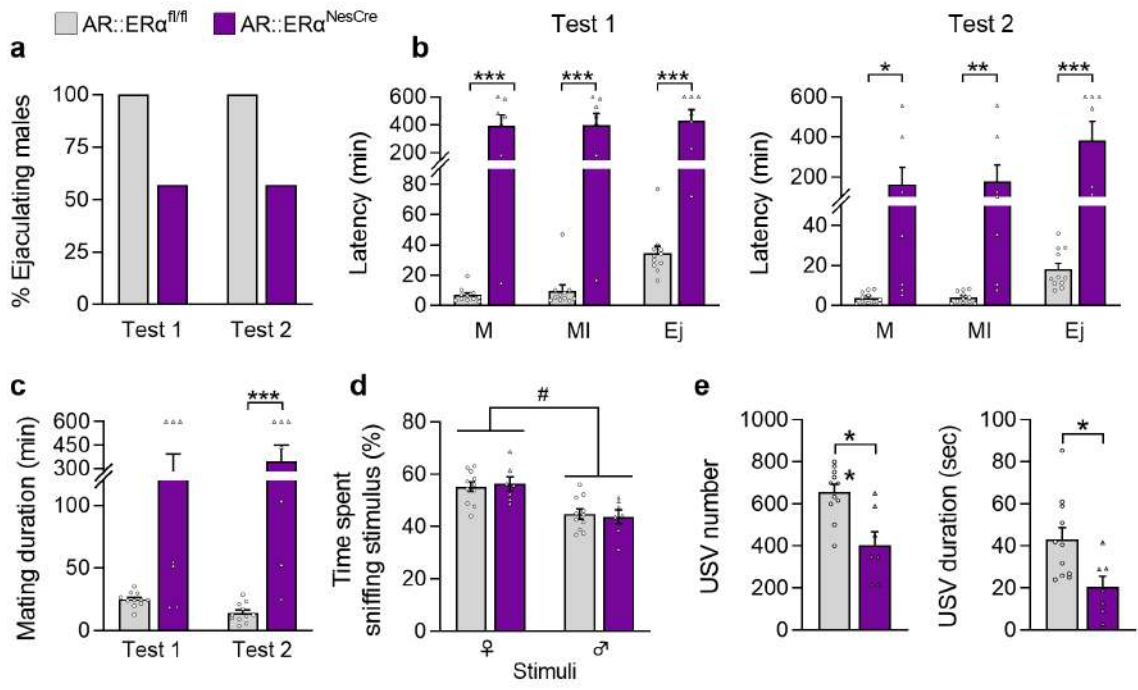
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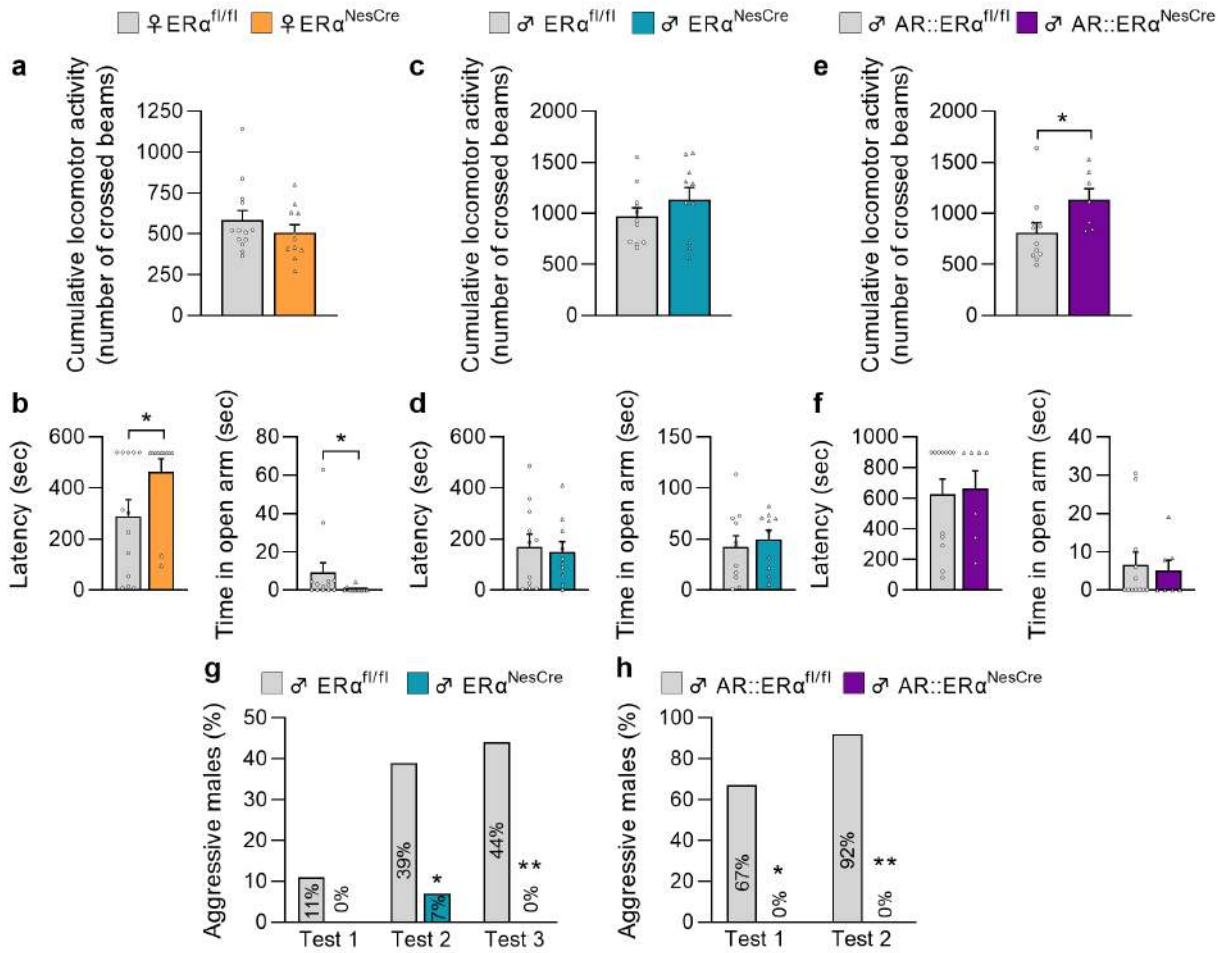
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785 **SUPPLEMENTARY INFORMATION**

786 **Content:**

787 Supplementary Figure 1, relative to Figure 1

788 Supplementary Figure 2

789 Supplementary Figure 3

790 Supplementary Figure 4

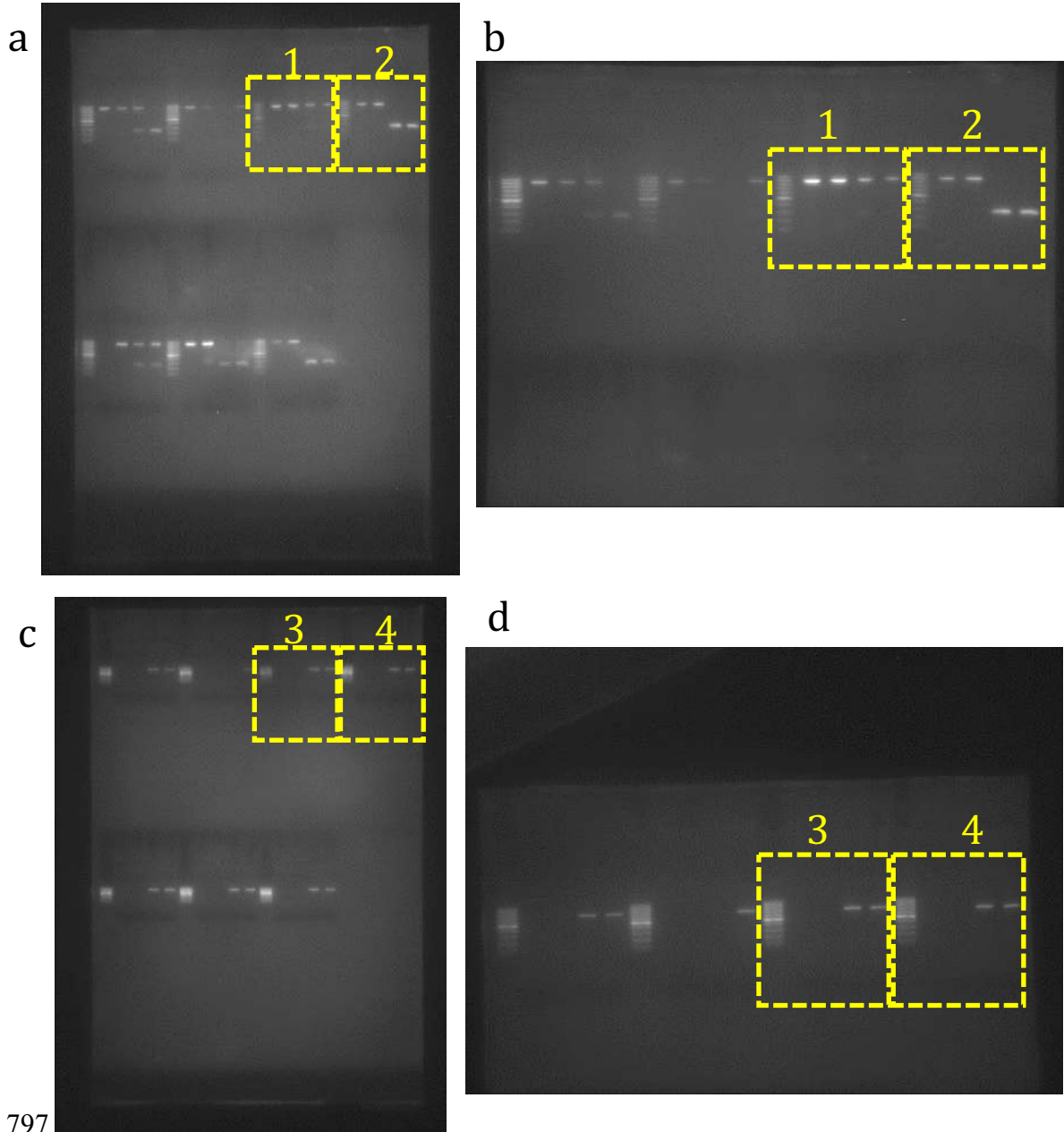
791 Supplementary Table 1

792 Supplementary Table 2

793

794

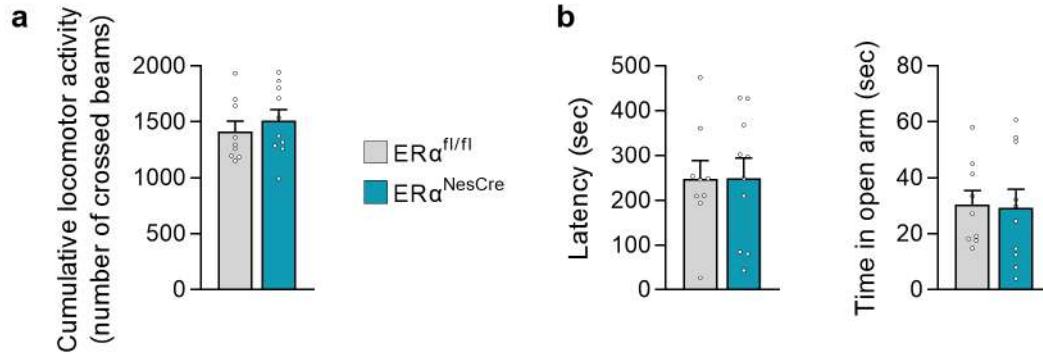
795  
796 **Supplementary Figure 1, relative to Figure 1.Uncropped gels.**



797  
798 The original gel (a and c) and the original gel magnification (b and d) used to construct the figure  
799 1a. The square labeled 1 correspond the upper left panel, the square labeled 2 to the upper right  
800 panel, the square labeled 3 to the lower left panel, and the square labeled 4 to the lower right panel.

801  
802

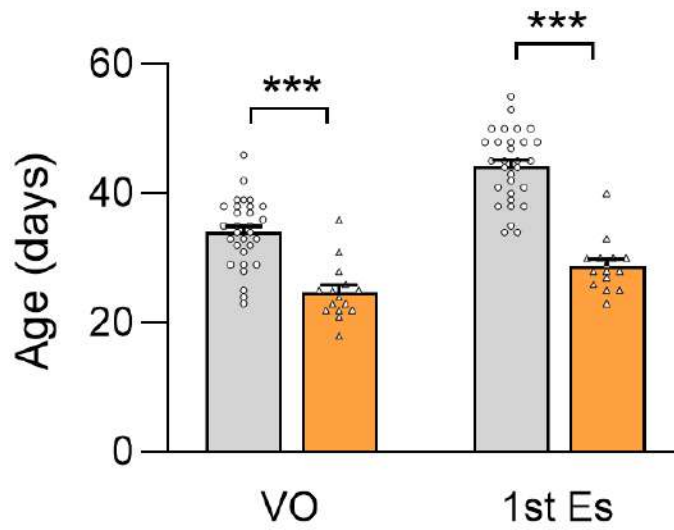
803  
804 **Supplementary Figure 2. Assessment of locomotor activity and anxiety-related behavior in**  
805 **gonadectomized and testosterone-supplemented ER<sup>NesCre</sup> males.**



806  
807  
808 **a.** Locomotor activity during the 2 h-test for control (ER $\alpha^{fl/fl}$ ) and mutant (ER $\alpha^{NesCre}$ ) males (n = 9-  
809 10 per genotype). **b. Left.** Latency to enter the open arms of the O-maze. **Right.** Time spent in the  
810 open arms. All data are shown as means  $\pm$  S.E.M with individual values.

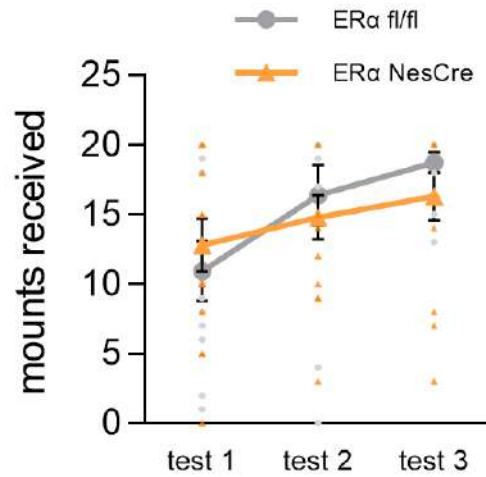
811  
812

813  
814 **Supplementary Figure 3. Delayed puberty onset in ER<sup>NesCre</sup> females.**



815  
816 Age at vaginal opening (VO) and first estrus (1<sup>st</sup> Es) of ER<sup>fl/fl</sup> and ER<sup>NesCre</sup> females (n = 15-30 per  
817 genotype). \*\*\*p < 0.001 versus control (ER<sup>fl/fl</sup>) animals. Data are shown as means ± S.E.M with  
818 individual values.  
819

820  
821 **Supplementary Figure 4. Behavior of male stimuli during the lordosis behavior assessment of**  
822 **ER<sup>NesCre</sup> females.**



823  
824 Number of mounts received during the three lordosis behavior tests of ER<sup>fl/fl</sup> and ER<sup>NesCre</sup> females (n  
825 = 11-13 per genotype). Male stimuli gave a similar amount of mounts to control and mutant  
826 females. Data are shown as means  $\pm$  S.E.M with individual values.  
827



828

829 **Supplementary Table 1. Analysis of physiological parameters in (ER $\alpha^{fl/fl}$ ) and mutant**830 **(ER $\alpha^{NesCre}$ ) females and males.**

| Genotype |                                   | ER $\alpha^{fl/fl}$ | ER $\alpha^{NesCre}$ |
|----------|-----------------------------------|---------------------|----------------------|
| Females  | Body weight (bw) (g)              | 23.99 $\pm$ 0.62    | 22.58 $\pm$ 0.49     |
|          | Ovary weight (%bw)                | 0.04 $\pm$ 0.001    | 0.03 $\pm$ 0.002**   |
|          | Uterus weight (%bw)               | 0.27 $\pm$ 0.01     | 0.73 $\pm$ 0.07***   |
|          | Estradiol levels (pg/ml)          | 46.61 $\pm$ 7.58    | 75.29 $\pm$ 9.28*    |
|          | Number of corpora lutea           | 2.45 $\pm$ 0.53     | 0                    |
| Males    | Body weight (bw) (g)              | 30.72 $\pm$ 0.74    | 29.04 $\pm$ 0.81*    |
|          | Seminal vesicle weight (%bw)      | 1.04 $\pm$ 0.05     | 1.25 $\pm$ 0.05**    |
|          | Testis weight (%bw)               | 0.65 $\pm$ 0.01     | 0.65 $\pm$ 0.02      |
|          | Testosterone levels (ng/ml)       | 5.51 $\pm$ 1.71     | 11.01 $\pm$ 1.52*    |
|          | Estradiol levels (pg/ml)          | 13.86 $\pm$ 1.95    | 8.39 $\pm$ 1.67*     |
|          | Sperm Count (10 <sup>6</sup> /mL) | 74.95 $\pm$ 9.27    | 77.31 $\pm$ 9.28     |

831 Data are means  $\pm$  S.E.M. for 11-12 females per genotype for body and organs weights, 7-9 females

832 per genotype for estradiol levels, 3 females per genotype for the number of corpora lutea, 22 to 24

833 males per genotype for body, seminal vesicles and testis weights, 8-11 males per genotype for

834 testosterone levels, 10-13 males per genotype for estradiol levels and 8 to 10 males per genotype for

835 sperm count. \*p < 0.05, \*\*p < 0.01; \*\*\*p < 0.001 versus control (ER $\alpha^{fl/fl}$ ) animals.

836

837

838

839  
840 **Supplementary Table 2. Analysis of reproductive parameters of control (AR::ER $\alpha^{fl/fl}$ ) and**  
841 **mutant (AR::ER $\alpha^{NesCre}$ ) males.**

| <b>Genotype</b>              | <b>AR::ER<math>\alpha^{fl/fl}</math></b> | <b>AR::ER<math>\alpha^{NesCre}</math></b> |
|------------------------------|------------------------------------------|-------------------------------------------|
| Body weight (bw) (g)         | 31.67 $\pm$ 1.31                         | 26.97 $\pm$ 0.69*                         |
| Seminal vesicle weight (%bw) | 0.95 $\pm$ 0.03                          | 1.49 $\pm$ 0.18***                        |
| Testis weight (%bw)          | 0.52 $\pm$ 0.04                          | 0.42 $\pm$ 0.04                           |
| Testosterone levels (ng/ml)  | 2.36 $\pm$ 0.57                          | 4.71 $\pm$ 0.91*                          |

842  
843 Data are means  $\pm$  S.E.M. for 10-17 males per genotype for body and organ weight and 8-10 for  
844 testosterone levels. \*p < 0.05, \*\*\*p < 0.001 versus control (ER $\alpha^{fl/fl}$ ) animals.

845