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1 **Title**

2 Effects of minipuberty disruption on the expression of sexual behavior in female mice

3

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16

17 **Keywords**

18 Minipuberty; Females; Sexual differentiation; Reproductive behaviors, Sex steroids

19 **Abstract**

20 Sex steroids influence early organization of neural structures involved in expression of sexual  
21 behaviors. A critical perinatal period during which testosterone surges occur has been identified in  
22 male rodents. Data are lacking for females, whose ovarian activity starts later in the postnatal period.  
23 Minipuberty has been described in female as a first postnatal period of gonadotropic axis activation.  
24 We transiently disrupted the minipubertal period, using daily injections of GnRH antagonist or  
25 aromatase inhibitor. The results showed unchanged puberty initiation and mild disturbance of estrus  
26 cyclicity. Minor modifications of some components of sexual behavior (olfactory preference, ability to  
27 attract male) were observed, but had no impact on lordosis behavior. Interestingly, letrozole-treated  
28 female mice showed increased anxiety state level at adulthood. This period therefore does not seem to  
29 be a key organizational window for female sexual behavior but appears to participate in the  
30 organization of neural structures involved in anxiety-related behavior.

## 31 **Introduction**

32 Sexual reproduction relies on optimal display of sexually dimorphic behaviors expressed by sexual  
33 partners. These include in rodents i) the emission and sensing of olfactory and auditory cues, which  
34 inform on the hormonal and receptive state and attract the partner, and ii) the postures adopted  
35 afterward to ensure insemination, such as lordosis behavior for females, mounts, thrusts and  
36 intromissions for males<sup>1-3</sup>.

37 Sex steroid hormones play a key role in the early organization of the neural structures involved in the  
38 expression of male and female behaviors. These processes have been extensively studied for half a  
39 century in males. However, the precise developmental period during which sex steroids act in females  
40 still needs to be documented. In rodents, it is indeed well established, since the seminal work of Phoenix  
41 et al.<sup>4</sup>, that testosterone released during the perinatal period by fetal and neonatal testes and its neural  
42 metabolite, estradiol, induce a permanent organization of the neural circuitry controlling male sexual  
43 behavior<sup>5</sup>. This consists in a potentiation of male (masculinization) and an inhibition of female  
44 (defeminization) behavioral characteristics, resulting in the expression of male-typical behaviors and  
45 the inability to adopt the female posture (lordosis).

46 In female rodents, there was a long-standing view that feminization of the neural circuitries regulating  
47 fertility and reproductive behaviors might occur as a default pathway. This idea stems from the fact  
48 that rodent ovaries do not produce sex steroids during the fetal and perinatal periods<sup>6</sup>. At these stages,  
49 the female brain is also protected from the masculinizing effects of maternal (and male sibling)  
50 estradiol through its selective binding to the  $\alpha$ -fetoprotein<sup>7</sup>. In mice, ovaries start releasing estradiol  
51 around postnatal day (PND) 7 and then rise to reach a maximum at puberty. The prepubertal increase  
52 of estradiol has been shown to participate in brain feminization<sup>8,9</sup>. For instance, constitutive deletion  
53 of *cyp19a1* gene, encoding the aromatase that metabolizes testosterone into estradiol, has been shown  
54 to reduce lordosis behavior in adult mutant female mice<sup>8</sup>. In these mice, estradiol supplementation from

55 PND15 to PND25 partially restored the behavioral deficiency, while earlier administration of estradiol  
56 between PND5 and PND15 did not<sup>9</sup>. These studies suggested that the period lasting from PND15 to  
57 PND25 may represent one of the key postnatal periods involved in the feminization of the neural  
58 circuitry underlying female sexual behavior. However, these *cyp19a1* knockout mice are depleted of  
59 estrogens from the embryonic period. They also present high levels of testosterone, which could  
60 directly or indirectly interfere with neural processes in the female brain.

61 More recently, high levels of estradiol were detected in the serum of female mice between PND12 and  
62 PND17, resulting from a massive and transient gonadotropin surge<sup>10</sup>. This minipubertal hypothalamic-  
63 pituitary-gonadal (HPG) activity may regulate uterus and mammary gland development but its role in  
64 female reproductive function remains unknown<sup>11</sup>.

65

66 The present study aims to determine the precise role of the minipubertal period in the organization  
67 of the neural circuits controlling female reproductive function and sexual behavior. For this purpose,  
68 we used two pharmacological models to transiently disrupt the ovarian production of estrogens during  
69 minipuberty. In the GonadoSTOP model, C57BL/6J female mice were injected from PND10 to PND16  
70 with a GnRH antagonist (Ganirelix®) to alter the gonadotropic axis. Such treatment was shown to  
71 suppress gonadotropin release and reduce estradiol levels by half after only two days of treatment<sup>10,12</sup>.  
72 In the second model, C57BL/6J female mice were treated from PND10 to PND17 with letrozole an  
73 inhibitor of aromatase, the enzyme that metabolizes testosterone into estradiol. As aromatase blockade  
74 increases the levels of testosterone, we added two other groups treated with letrozole and flutamide (an  
75 androgen receptor antagonist) or with flutamide alone. For both models, we monitored body weight,  
76 assessed puberty initiation (age at vaginal opening and first estrus) and analyzed estrous cyclicity in  
77 adulthood. Afterwards, female mice were ovariectomized and primed with estradiol and progesterone  
78 to measure their sexual behavior under normalized hormonal levels. We performed tests for olfactory

79 preference, ability to attract the male partner and lordosis behavior. In addition, general behavior,  
80 including locomotor activity and anxiety-related behavior, was assessed.

## 81 **Results**

### 82 **Effects of minipubertal treatment with Ganirelix<sup>®</sup> (Experiment 1)**

#### 83 *Pubertal onset, estrous cyclicity and other physiological parameters*

84 Postnatal C57BL6/J females were injected with vehicle or Ganirelix for 7 days from PND10 to PND16  
85 (Figure 1). The ages at vaginal opening and first estrus were monitored for both groups. The results in  
86 figure 2A-B shows that there were no differences between the control and Ganirelix-treated females  
87 for the age at vaginal opening ( $p = 0.84$ ) and first estrus ( $p = 0.44$ ). The duration between vaginal  
88 opening and first estrus was also comparable between the two treated groups ( $p = 0.15$ ; Figure 2C).

89 In adult females, we monitored the estrous cycle for 5 weeks. One vehicle and 5 Ganirelix-treated  
90 females showed irregular cycles and were not included in the detailed analysis. A Fisher's exact test  
91 indicated no effect of the treatment on this parameter ( $p = 0.18$ ). Further analysis showed that the  
92 treatment did not affect the mean cycle duration ( $p = 0.75$ ; Figure 2D). Detailed analysis of the estrous  
93 cycle (Figure 2E) showed a comparable duration of the estrus and diestrus phases between controls  
94 and Ganirelix-treated females ( $p = 0.45$ ,  $p = 0.68$ ; respectively). In contrast, the metestrus phase was  
95 shorter by 13.6% in the Ganirelix group ( $p = 0.0228$ ), while a tendency to increase was observed for  
96 the proestrus phase ( $p = 0.077$ ).

97 Females from vehicle- and Ganirelix-treated mice were weekly monitored for their body weight until  
98 ovariectomy. This showed no effect of the treatment ( $F_{(1, 26)} = 0.57$ ,  $p = 0.45$ ), but an effect of age  
99 ( $F_{(1.317, 34.25)} = 668.6$ ,  $p < 0.0001$ ) (Figure 2F). There also was no effect on body weight at vaginal  
100 opening ( $p = 0.68$ ) (Figure S3A).

101 The ovaries collected from controls and Ganirelix-treated adult females at the time of ovariectomy  
102 were weighed. No difference between the two groups was observed ( $0.024\% \pm 0.002$  of body weight  
103 in controls versus  $0.027\% \pm 0.001$  of body weight in Ganirelix-treated females;  $p = 0.45$ ) (Figure S3B).

104

#### 105 *Behavior*

106 Sexual and general behaviors were assessed in vehicle- and Ganirelix-treated females, which were  
107 ovariectomized at the end of estrous cyclicity monitoring and supplemented with estradiol and  
108 progesterone.

#### 109 *Sexual behavior*

110 The ability of females to discriminate between male and female pheromones was tested in a Y-maze  
111 with male and female stimuli, both anesthetized and placed at each end of the maze (Figure 3A). The  
112 total time spent in chemo-investigation was not different between the vehicle- and Ganirelix-treated  
113 females ( $48.35 \pm 3.11$  s versus  $57.66 \pm 5.88$  s;  $p = 0.1751$ ) (Figure S1A). Two-way ANOVA indicated  
114 no effect of stimulus ( $F_{(1, 48)} = 0.52$ ;  $p = 0.47$ ) and treatment ( $F_{(1, 48)} = 0.005211$ ,  $p = 0.9428$ ) on the  
115 number of entries into the arms (Figure S1B). The calculated discrimination index showed a preference  
116 towards male for the vehicle-treated females ( $p = 0.0363$ ; Figure 3B). Although positive, the  
117 discrimination index calculated for Ganirelix-treated females did not reach the statistical significance  
118 ( $p = 0.0678$ ). Comparison of the two treated groups did not show differences ( $p = 0.35$ ).

119 In mating tests, females were tested in the presence of a sexually experienced male for 30 min (Figure  
120 3C). Males exhibited a comparable number of mounts in the presence of vehicle- or Ganirelix-treated  
121 females ( $p = 0.20$ ) as shown in Figure 3D. The lordosis quotient did not differ between the two groups  
122 of treated females ( $p = 0.47$ ; Figure 3E).

123 The ability of vehicle- and Ganirelix-treated females to attract sexually experienced males was assessed  
124 using the three-chamber test (Figure 3F). The total time spent in investigation ( $p = 0.94$ ; Figure S1E),  
125 the number of entries into the stimulus compartments ( $p = 0.42$ ; Figure S1F) and the percentage of  
126 time spent by males in the investigation of each stimulus ( $p = 0.12$ ; Figure 3G) were comparable  
127 between controls and Ganirelix-treated females.

128 In the Y-maze test, vehicle- and Ganirelix-treated females were replaced by their urine (Figure 3H).  
129 As for the three-chamber test, the total time spent in stimulus arms ( $p = 0.78$ ; Figure S1I), the number  
130 of entries into the two arms ( $p = 0.58$ ; Figure S1J) and the time spent in the investigation of each  
131 stimulus ( $p = 0.39$ ; Figure 3I) were comparable between vehicle- and Ganirelix-treated females.

### 132 *General behaviors*

133 Locomotor activity of both treated groups was assessed in a circular corridor for 2 h. Two-way  
134 ANOVA showed an effect of time ( $F_{(3,337, 83.43)} = 68.13$ ;  $p < 0.0001$ ) but not of treatment ( $F_{(1, 25)} = 0.47$ ,  
135  $p = 0.50$ ) (Figure 6A). Comparison of cumulative activity over the 2 hours of the test did not show  
136 differences between the two groups ( $p = 0.50$ ) (Figure 6B).

137 Anxiety-related behavior was analyzed in an 0-maze paradigm (Figure 6E). No difference was  
138 observed between the two groups in the latency to the first entry into the open arm ( $p = 0.61$ ; Figure  
139 6F), or the number of entries ( $p = 0.89$ ; Figure 6G). The time spent in the open arms was also  
140 comparable between the treated groups ( $p = 0.38$ ; Figure 6H).

### 141 *ER $\alpha$ -immunoreactivity*

142 The brains of female mice that were subjected to behavioral tests were analyzed for ER $\alpha$ -  
143 immunostaining. It was performed in keys hypothalamic regions controlling female sexual behavior,  
144 including the activating nuclei VMH and the inhibitory regions, ARC and POA<sup>13,14</sup>. Figure S2 shows

145 a specific nuclear staining in the POA, VMH and ARC. Quantification of the number of ER $\alpha$ -  
146 immunoreactive cells showed no difference between vehicle- and Ganirelix-treated females in the POA  
147 ( $p = 0.29$ ), VMH ( $p = 0.78$ ) and ARC ( $p = 0.97$ ) (Figure S2A-D).

148

149 **Effects of separate and combined treatment with letrozole and flutamide during minipuberty**  
150 **(Experiment 2)**

151 Treatment of female mice with Ganirelix from PND10 to PND16 did not affect the expression of sexual  
152 behavior. As Ganirelix treatment only partially suppresses the ovarian production of estradiol<sup>10</sup>, it is  
153 possible that the remaining estradiol was sufficient to induce the organization of the neural circuitry  
154 underlying female sexual behavior. Therefore, in Experiment 2, C57BL6/J females were daily injected  
155 for 8 days from PND10 to PND17 with vehicle (control group), letrozole alone at 50  $\mu$ g/day to inhibit  
156 aromatase activity and thus estradiol production, flutamide alone at 0.1 mg/day, or with both  
157 compounds (Figure 1). The anti-androgen flutamide was used to counteract the androgenic effects  
158 potentially induced by the known elevation of testosterone levels triggered by the blockade of  
159 aromatase and thus the metabolization of testosterone into estradiol.

160 *Pubertal onset, estrous cyclicity and other physiological parameters*

161 Monitoring of pubertal onset markers showed no effect of the treatment on the age at vaginal opening  
162 ( $p = 0.54$ ), while an effect of treatment was observed on the age at first estrus ( $p = 0.018$ ) (Figure 4A-  
163 B). *Post hoc* analysis indicated that females treated with both letrozole/flutamide had the first estrus  
164 4.14 days later than those treated with flutamide ( $p = 0.046$ ; Figure 4B). Analysis of the interval  
165 between vaginal opening and first estrus did not show any effect of the treatment ( $p = 0.27$ ; Figure 4C).

166 We monitored the estrous cycle for 5 weeks in adult females. One vehicle, one flutamide and 4  
167 letrozole/flutamide-treated females showed irregular cycles and were not included in the detailed

168 analysis. A Fisher's exact test indicated no effect of the treatment on this parameter ( $p = 0.072$ ). The  
169 mean duration of the estrous cycle was unaffected by the treatment ( $p = 0.1317$ ; Figure 4D). Detailed  
170 analysis of each cycle phase duration revealed no effect of the treatment on the duration of the proestrus  
171 and estrus and metestrus phases ( $p = 0.29$ ,  $p = 0.99$ ,  $p = 0.06$ ; respectively), although the statistical  
172 significance was close to 0.05 for the metestrus phase. In contrast, an effect of treatment was observed  
173 for the diestrus phase ( $p = 0.023$ ). Females treated with letrozole/flutamide had a longer duration  
174 compared to those treated with flutamide (+ 31.77%;  $p = 0.046$ ) (Figure 4E).

175 For body weight monitored between PND20 and PND90, two-way ANOVA showed effects of age  
176 ( $F_{(1.652, 66.08)} = 1545$ ,  $p < 0.0001$ ) and treatment ( $F_{(3, 40)} = 3.826$ ,  $p = 0.017$ ) (Figure 4F). *Post hoc* analysis  
177 pointed out a transient increase of body weight at PND21 for letrozole-treated females ( $p = 0.015$ ) and  
178 letrozole/flutamide-treated groups ( $p = 0.023$ ) compared to the vehicle-treated females, and at PND70  
179 for the letrozole group compared to females treated with flutamide ( $p = 0.027$ ). There also was no  
180 effect on body weight at vaginal opening ( $p = 0.095$ ) (Figure S3C).

181 The weight of ovaries, collected at the time of ovariectomy, was not different between the treated  
182 groups ( $p = 0.13$ ) as illustrated in Figure S3D.

### 183 *Behavior*

184 Sexual behavior was analyzed in vehicle-, flutamide-, letrozole- and letrozole/flutamide-treated mice,  
185 which were ovariectomized at the end of estrous cyclicity monitoring and hormonally primed as  
186 described above.

#### 187 *Sexual behavior*

188 In the olfactory preference test (Figure 5A), there was no effect of the treatment on the total time spent  
189 in chemoinvestigation for the four female-treated groups ( $p = 0.1689$ ; Figure S1C). Similarly, no effect

190 of the treatment ( $F_{(3, 86)} = 0.7207$ ;  $p = 0.5423$ ) and stimulus ( $F_{(1, 86)} = 0.8275$ ;  $p = 0.3655$ ) was observed  
191 for the number of entries into the arms (Figure S1D). All females exhibited preference for the male  
192 stimulus ( $p = 0.007$ ,  $p = 0.010$ ,  $p = 0.036$ ,  $p = 0.006$ , for vehicle-, flutamide-, letrozole- and  
193 letrozole/flutamide-treated mice; respectively). One-way ANOVA showed no effect of the treatment  
194 on the discrimination index ( $p = 0.98$ ; Figure 5B).

195 In the mating test, sexually experienced males exhibited comparable number of mounts in the presence  
196 of females ( $p = 0.69$ ), regardless of their treatment (Figure 5C-D). Furthermore, no effect of the  
197 treatment was observed on the lordosis quotient ( $p = 0.72$ ; Figure 5E).

198 In the three-chamber paradigm (Figure 5F), there were no differences in the total time spent by males  
199 in the stimulus compartments ( $p = 0.55$ ,  $p = 0.72$ ,  $p = 0.19$ , for vehicle versus flutamide groups, vehicle  
200 versus letrozole groups and vehicle versus letrozole/flutamide groups, respectively, Figure S1G), nor  
201 in the number of entries in the compartments ( $p = 0.49$ ,  $p = 0.32$  and  $p = 0.31$  for vehicle versus  
202 flutamide groups, vehicle versus letrozole groups and vehicle versus letrozole/flutamide groups,  
203 respectively) (Figure S1H). The percentage of time spent by males investigating vehicle- versus  
204 flutamide-treated females ( $p = 0.59$ ) and vehicle- versus letrozole-treated females ( $p = 0.8$ ) was not  
205 different. In contrast, males spent less time investigating letrozole/flutamide-treated mice than controls  
206 (-29.37%,  $p = 0.009$ ) as shown in (Figure 5G).

207 For urinary preference (Figure 5H), the total time spent in the stimulus arms was not different ( $p =$   
208  $0.41$ ,  $p = 0.59$ ,  $p = 0.59$ , for vehicle versus flutamide groups, vehicle versus letrozole groups and vehicle  
209 versus letrozole/flutamide groups, respectively; Figure S1K). The number of entries into each arm was  
210 also comparable between the three experimental conditions ( $p = 0.45$ ,  $p = 0.83$  and  $p = 0.68$  for vehicle  
211 versus flutamide groups, vehicle versus letrozole groups and vehicle versus letrozole/flutamide groups,  
212 respectively; Figure S1L). In addition, the percentage of time spent by males in investigation was not

213 different between the two stimuli for the three experimental conditions ( $p = 0.18$ ,  $p = 0.39$ ,  $p = 0.46$   
214 for vehicle versus flutamide groups, vehicle versus letrozole groups and vehicle versus  
215 letrozole/flutamide groups, respectively; Figure 5I).

### 216 *General Behaviors*

217 Analysis of locomotor activity by two-way ANOVA showed an effect of time ( $F_{(3,225, 129.0)} = 87.56$ ,  $p$   
218  $< 0.0001$ ) but not of treatment ( $F_{(3, 40)} = 0.598$ ,  $p = 0.62$ ) (Figure 6C). There was no effect of the  
219 treatment on the cumulative activity over the 2 hours of the test ( $p = 0.76$ ; Figure 6D).

220 For anxiety-related behavior, there was no effect of the treatment on the latency to the first entry into  
221 the open arm ( $p = 0.77$ , Figure 6I), but a tendency to decrease was observed for the number of entries  
222 although it did not reach the statistical significance ( $p = 0.059$ , Figure 6J). One-way ANOVA showed  
223 an effect of treatment on the time spent into the open arms ( $F_{(3, 43)} = 2.995$ ,  $p = 0.041$ ), with reduced  
224 time spent in the open arms for letrozole-treated mice compared to vehicle group ( $-48.51\%$ ,  $p = 0.0176$ )  
225 (Figure 6K).

## 226 **Discussion**

227 In this study, using two pharmacological methods, we investigated the impact of minipubertal  
228 disruption on later pubertal initiation, estrous cyclicity and female sexual behavior.

229 Disruption of the gonadotropic axis through GnRH antagonist administration (Ganirelix) during the  
230 minipubertal period did not impact the age of puberty initiation, with no change in the age at vaginal  
231 opening or first estrus in female mice. Adult monitoring of estrous cyclicity showed reduced duration  
232 of the metestrus phase in females treated with Ganirelix, without modification of the total duration of  
233 the estrous cycle. It has also been previously shown that minipubertal Ganirelix treatment induces no  
234 modification in fertility at 3 months of age, suggesting that the slight treatment effect observed on the  
235 duration of the metestrus phase does not appear to impact fertility in adulthood. Surprisingly, 11-  
236 month-old treated females showed an increase in the percentage of mice becoming pregnant,  
237 suggesting a long-term effect of the treatment on reproductive longevity (Chester et al., preprint  
238 available at bioRxiv<sup>15</sup>).

239 Regarding the treatment with an aromatase inhibitor (letrozole) in the absence or presence of an anti-  
240 androgen (flutamide), an effect of treatment was observed on the age at the first estrus. This difference  
241 was observed only between the flutamide and letrozole/flutamide groups with no difference compared  
242 with vehicles. In adulthood, an effect of treatment was observed on the duration of the diestrus phase  
243 of the estrous cycle. Again, difference was noticed between the flutamide and letrozole/flutamide  
244 groups. The meaning of such differences in the initiation of puberty and estrous cyclicity between these  
245 two groups is not clear. Interestingly, it is documented that androgens through the androgen receptor  
246 (AR) play a key role in both follicular development and female neural regulation of the gonadotropic  
247 axis<sup>16-19</sup>. It is therefore possible that both treatments affected ovarian function and/or gonadotropic axis  
248 differently, leading to the observed differences. Nevertheless, it is important to point out that the age  
249 of pubertal onset and the duration of the diestrus phase of the cycle in females treated with letrozole or  
250 flutamide alone or in combination were in the range of those observed for the vehicle group. In addition,

251 treatments with letrozole or letrozole/flutamide had slight effects on body weight at PND20, suggesting  
252 that these treatments, compared to Ganirelix, transiently affected the somatotropic axis.

253 At the behavioral level, analyses were performed in ovariectomized and hormonally primed females  
254 to test the direct effects of the minipubertal treatments on the neural circuitry underlying sexual  
255 behavior. Under such normalized hormonal levels, the discrimination index in the olfactory preference  
256 test was slightly affected by the treatment with Ganirelix. However, no significant difference was found  
257 with the vehicle group. Olfactory stimulation is mandatory for the activation of the other components  
258 of sexual behavior. Lordosis behavior, partner and urinary preferences were not affected by Ganirelix  
259 treatment. In agreement with these data, the number of ER $\alpha$ -immunoreactive cells was comparable  
260 between controls and Ganirelix-treated females in the neural circuitry underlying sexual behavior  
261 (VMH, POA and ARC).

262 Female mice treated during minipuberty with letrozole, flutamide or letrozole/flutamide showed  
263 comparable olfactory preference. In the three-chamber test, females of the letrozole/flutamide group  
264 attracted less the males compared to the control group. This effect was not observed in the urinary  
265 preference test, suggesting differences in the volatile cues between vehicle- and letrozole/flutamide-  
266 treated groups. Despite this reduced partner preference, mating assessed by the lordosis quotient was  
267 comparable between treatment groups.

268 Overall, this comprehensive behavioral analysis shows that under normalized hormonal levels, the  
269 expression of female sexual behavior was not or slightly impacted following minipubertal treatment  
270 with a GnRH antagonist or aromatase inhibitor, in the absence or presence of flutamide. This confirms  
271 a previous study showing that estradiol supplementation between PND5 and PND15 did not restore  
272 the expression of sexual behavior in aromatase knockout females<sup>9</sup>. In line with the classic view that  
273 neonatal estradiol masculinizes the brain structures involved in sexual behavior, this early postnatal  
274 treatment masculinized aromatase knockout females similarly to controls, which both exhibited

275 female-directed (male typical) mounting behavior when treated with testosterone in adulthood<sup>9</sup>. In the  
276 same study, estradiol treatment from PND15 to PND25 partially restored the expression of lordosis  
277 behavior of aromatase knockout adult females. These and our data strongly support that the period  
278 involved in the feminization of the neural circuits underlying female sexual behavior occurs at a later  
279 age<sup>9</sup>. Further research will be instrumental to delineate the precise prepubertal/pubertal periods during  
280 which neural feminization takes place, and determine the underlying mechanisms of action.

281 In addition to sexual behavior, we assessed general behavior. The results showed no effect of the  
282 treatments on locomotor activity. In contrast, an increased anxiety state level was observed in female  
283 mice treated during the minipubertal period with letrozole. This effect was likely induced by the  
284 transient depletion of estradiol during the minipubertal period, since hormonal levels were normalized  
285 in adult females analyzed for this behavior. No such effect was observed with Ganirelix treatment,  
286 probably explained by the incomplete reduction in estradiol levels in this model. In previous studies,  
287 we have shown that deletion of estrogen receptor (ER) *ERβ* or *ERα* in neural progenitors since  
288 embryonic day 10 resulted in increased anxiety-related behavior in adult female mice<sup>20-22</sup>. The present  
289 data suggest that estradiol released during the minipubertal period (PND10 to PND17) participates in  
290 the organization of the neural structures involved in anxiety-related behavior. It is well known that  
291 estradiol in adulthood controls the anxiety state of females. Indeed, elevated level of estradiol during  
292 the follicular phase induce an anxiolytic effect<sup>23</sup>. Downstream molecular pathways may imply the  
293 serotonergic system and the expression of tryptophan hydroxylase (TPH) in the dorsal raphe  
294 nucleus<sup>24</sup>. For instance, the increase in anxiety-related behavior observed in females deleted for neural  
295 *ERβ* was associated with a reduction in TPH2-immunoreactivity in the dorsal raphe nucleus since  
296 PND30 (unpublished observation,<sup>22</sup>). It remains to be determined whether the estradiol released during  
297 the minipubertal period participates in the organization of this neural pathway.

298

299 *Perspective and significance*

300 In summary, the evaluation of the impact of minipubertal disruption on puberty initiation, estrous  
301 cyclicity and female sexual behavior has shown that estradiol released during this period appears to  
302 affect the development of female reproductive function and behavior in subtle ways but is not essential  
303 for successful fertility and mating in adulthood. A transient activation of the HPG axis during  
304 minipuberty, characterized by high levels of gonadotropin LH (luteinizing hormone) and FSH (follicle-  
305 stimulating hormone) and elevated gonadal sex steroid production, occurs also in humans shortly after  
306 birth and can last up to 3-4 years in girls<sup>25</sup>. The influence of this period on women fertility remains to  
307 be determined. Further studies focusing on later developmental periods (prepubertal/pubertal) will help  
308 identify the critical developmental periods for brain feminization of reproductive function. Meanwhile,  
309 interestingly, this study revealed that estradiol released during minipuberty may play a role in the  
310 organization of anxiety-related behaviors. This is consistent with the well-established role for estradiol  
311 in the regulation of women's anxiety throughout life, whether during the menstrual cycle, menopause  
312 or in post-partum anxiety disorders. A better understanding of the critical periods of estradiol action  
313 would enable better management of these anxiety troubles.

314

315 **Conclusions**

316 In this study, we showed that transient disruption of the minipubertal period, in female mice, induced  
317 only minor modifications of estrous cyclicity and some components of sexual behavior (olfactory and  
318 partner preferences) without impacting subsequent mating behavior. Interestingly, this study, however,  
319 revealed that estradiol released during minipuberty may contribute to the organization of female  
320 anxiety-related behavior. Hence, these findings support the view that estradiol acts at different periods  
321 of development to organize distinct behaviors. Further research will precise the windows of action and  
322 the underlying mechanisms of action.

## 323 **Methods**

### 324 **Animal and Treatments**

325 Female mice were obtained from local mating of male and female C57BL/6J mice (Janvier Labs).  
326 Animals were maintained at a temperature of 22°C and subjected to a 12-hour light-dark cycle with  
327 the dark phase beginning at 1:30 PM and ending at 1:30 AM. They had *ad libitum* access to both food  
328 (A03-10; SAFE) and water.

329

330 *Experiment 1.* Female mice were subcutaneously injected each day from PND10 to PND16 with 20 µl  
331 of sesame oil alone (control group) or 20 µl of sesame oil containing 10 µg of Ganirelix® (Fyremadel  
332 Gé, FERRING), a GnRH antagonist (n = 12-15 per treatment, 3 different litters for control- and 4 litters  
333 for Ganirelix-treated animals) (Figure 1A). Under such experimental conditions, Ganirelix treatment  
334 reduced estradiol levels by 50 % in female mice<sup>10</sup>.

335

336 *Experiment 2.* Female mice were subcutaneously injected each day from PND10 to PND17 with 20 µl  
337 of sesame oil and ethanol 3% for the vehicle group, or containing either 50 µg of letrozole (a reversible  
338 aromatase inhibitor), 0.1 mg of flutamide (a non-steroidal anti-androgen), or both (letrozole/flutamide)  
339 (n = 10-13 per treatment, 3 different litters for control- and 4 litters for letrozole-, flutamide- and  
340 letrozole/flutamide-treated animals) (Figure 1B). The concentrations of letrozole and flutamide were  
341 chosen based on previous studies in mice<sup>26,27</sup>. Administration of 50 µg per day of letrozole reduced  
342 estradiol level below the limit of assay sensitivity, as in ovariectomized females<sup>27</sup>. In a prenatally  
343 androgenized mouse model, daily injection of the same concentration of flutamide was efficient to  
344 block androgen receptor signaling and restore estrous cyclicity and GnRH neuron circuit integrity,  
345 while unaffected ovarian and adrenal gland weights<sup>26</sup>.

346

347 For both Experiments 1 and 2, female mice were weaned at PND28 and identified by numbers. No  
348 information on the treatment groups were available during the analyses.

### 349 **Pubertal onset, estrous cyclicity, body and organ weights**

350 The age at pubertal onset was monitored by daily examination of vaginal opening since PND20 (Figure  
351 1A-B). Once vaginal opening was observed, vaginal smears, flushed with physiological saline (NaCl  
352 0.9%), were collected daily to determine the age of first estrus. Vaginal smears were collected daily  
353 until PND90 to examine estrous cyclicity. Microscopic examination of the vaginal smears, following  
354 hematoxylin/eosin staining, allowed identification of the estrous cycle phases. The mean duration of  
355 the estrous cycle and the number of days spent in each stage were quantified.

356 Body weight was assessed from PND21 to PND90. The ovaries were collected and weighed at the time  
357 of ovariectomy two weeks before the beginning of behavioral assessment.

358

### 359 **Behavioral tests**

360 Experiments were carried out under red-light illumination, occurring 2 h after lights were turned off.  
361 All sessions were recorded via videotaping for subsequent analysis. The testing sequence was  
362 proceeded as following: 1<sup>st</sup> test of lordosis behavior for sexual experience, olfactory preference test,  
363 locomotor activity monitoring, 2<sup>nd</sup> test of lordosis, 0-maze test, three-chamber test for partner  
364 preference and Y-maze for urinary preference. Behavioral tests were separated by at least two full days  
365 of complete rest or habituation to the following test. The stud males used were sexually experienced  
366 three times before the beginning of behavioral tests. All devices used in these tests, except for the  
367 animal home cages, were cleaned with a solution of 10% ethanol between trials.

368

369 *Preparation of females*

370 Female mice were ovariectomized two weeks before the beginning of behavioral tests under general  
371 anesthesia and received a 1 cm subcutaneous SILASTIC™ filled with 50 µg of estradiol benzoate  
372 (Sigma-Aldrich) diluted in 30 µL sesame oil and sealed at each end with SILASTIC™ adhesive as  
373 previously described<sup>20,28,29</sup>. Five hours before each behavioral test, females were primed with a  
374 subcutaneous injection of progesterone (1 mg/100 µL).

375

### 376 *Lordosis*

377 Each sexually-experienced female was introduced into the cage of a sexually experienced male. The  
378 duration of each test was set to 30 min. The female was considered in a lordosis posture when, in  
379 response to male mount, all four paws made contact with the ground, the hind section lifted while the  
380 back region formed an arch. The number of male mounts, the percentage of females displaying lordosis  
381 behavior and the lordosis quotient of each female were calculated. The lordosis quotient was calculated  
382 as the number of lordosis postures divided by the number of male mounts as previously described<sup>20,28</sup>.

383

### 384 *Olfactory Preference*

385 Olfactory preference of female mice was tested in an enclosed plexiglass Y-maze, following a  
386 previously established protocol<sup>20,30,31</sup>. The maze comprised 3 equivalent arms (25.8 cm length, 11 cm  
387 width, 30.7 cm height). Female mice underwent a familiarization period over two consecutive days  
388 (day 1 and 2), during which two empty perforated goal boxes were positioned at each end of two arms  
389 for a duration of 10 min. On the testing day (day 3), females were placed in the empty arm and had  
390 choice for 10 min between a sexually receptive female and an intact male, both placed within the goal  
391 boxes in the two opposing arms. To prevent social interaction, the stimuli were anesthetized.  
392 Observations included the time spent in chemo-investigation of the stimuli and the number of entries

393 into each arm of the maze. The discrimination index was calculated as the difference in time spent  
394 investigating the male (M) versus the female (F), divided by the total investigation time.

395

### 396 *Partner Preference tests*

#### 397 *Three-chamber test*

398 Sexually experienced males underwent a familiarization phase with the testing arena, a three-  
399 chambered box (22.5 cm height, 40.5 cm width, and 62.4 cm length; 20.5 cm for the two peripheral  
400 chambers and 21.4 cm for the central chamber) for 10 min over two consecutive days<sup>30,31</sup>. During this  
401 period, two empty perforated goal boxes were placed within the side chambers of the arena. On the test  
402 day, each male was introduced into the neutral chamber and allowed to freely explore for 10 min. A  
403 vehicle-treated female was put in a goal box, while the opposite one contained a female treated with  
404 Ganirelix (Experiment 1), flutamide, letrozole or flutamide/letrozole (Experiment 2). Females were  
405 randomly assigned to either the left or right chamber. The number of entries into each compartment  
406 and the duration the male spent sniffing each female were analyzed.

407

#### 408 *Y-Maze test (Urinary Preference)*

409 Sexually experienced males underwent a familiarization period with the maze for 10 min over two  
410 consecutive days<sup>30,31</sup>. On the testing day, males had choice between urine of females from the vehicle  
411 group and urine from one of the treated groups, Ganirelix (Experiment 1) or flutamide, letrozole or  
412 flutamide/letrozole (Experiment 2), both put inside perforated goal boxes placed at each end of the  
413 maze. Urine was collected one hour prior to the test. Urine samples from all females within each  
414 treatment group were combined in equal volumes, and 10  $\mu$ L of this mixture was applied onto a piece  
415 of filter paper. Observations were made on the time spent by males chemo-investigating each stimulus  
416 and on the number of entries into each arm of the maze.

417

418 *Elevated 0-maze*

419 The elevated 0-maze consisted in a 56 cm diameter circular track (5 cm width), divided into two open  
420 and two enclosed (bordered by 17 cm-diameter walls) quadrants, elevated at 65 cm above the ground.  
421 Female were placed in one of the enclosed quadrants and allowed to freely explore the maze for 9  
422 minutes. Anxiety-like behavior was assessed by measuring the latency to the first entry into the open  
423 arms, the number of entries and the time spent in the open arms. A mouse was considered to be in an  
424 open arm when the four paws have entered.

425

426 *Locomotor activity*

427 The circular corridor used for activity measurement consisted in two concentric cylinders crossed by  
428 four diametrically opposed infrared beams<sup>29,30</sup>. Locomotor activity was recorded each time animals  
429 interrupted two successive beams, indicating they had traveled a quarter of the circular corridor. The  
430 activity monitored every 20 min and cumulative activity over the 120-min test were reported.

431

432 **Immunohistochemistry**

433 For experiment 1, three days after the end of behavioral assessment, females were euthanized 4 to 5 h  
434 after progesterone injection through intraperitoneal administration of ketamine (100 mg/kg) and  
435 xylazine (10mg/kg). They were intracardially perfused with NaCl (0.9%) followed by 4%  
436 paraformaldehyde (PFA) in Phosphate Buffer (PB). The brains were extracted, post-fixed overnight in  
437 4% PB-PFA, then frozen in tissue freezing medium (Leica) and stored at -20°C. The brains were then  
438 sectioned into 30 µm coronal sections using a cryostat (Leica CM 3050S).

439 For immunodetection, the sections were incubated in a solution containing 1X Phosphate Buffer Saline  
440 (PBS) - 0.3% Triton with 2% normal donkey serum for 1 h to saturate non-specific binding sites.  
441 Incubation with the primary antibody anti-ER $\alpha$  (1:400; Santa Cruz Biotechnology; rabbit) was  
442 performed in 1X PBS - 0.3% Triton for 3 days at 4°C. The sections were washed then incubated with  
443 chicken secondary anti-rabbit coupled with Alexa Fluor 488 (1:500; Invitrogen) in 1X PBS - 0.3%  
444 Triton for 2 h at room temperature in the dark. The sections were washed, mounted on SuperFrost  
445 slides, dried in the dark at room temperature, and then cover-slipped with Mowiol.  
446 The slides were scanned using a Nanozoomer Hamamatsu Scanner (Hamamatsu corporation) at high  
447 resolution and analyzed using NDP.view software. The regions of interest, VMH, arcuate nucleus  
448 (ARC), and preoptic area (POA), were identified using the Paxinos & Franklin Mouse Brain in  
449 Stereotaxic Coordinates Third Edition (2007) (plate 46, plate 43 and plate 30 respectively). After  
450 delineating equivalent area zones (0.3 mm<sup>2</sup>, 0.16 mm<sup>2</sup>, and 0.4 mm<sup>2</sup>, respectively), ER $\alpha$ -  
451 immunoreactive neurons were counted in the left and right hemispheres.

452

### 453 **Statistics**

454 The data are expressed as the mean  $\pm$  standard error of the mean. A *p*-value < 0.05 was considered  
455 significant. Normality of the data was assessed with the Shapiro-Wilks normality test. Unpaired t-test  
456 and Mann-Whitney test were used to compare vehicle- and ganirelix- treated mice (Experiment 1) for  
457 vaginal opening, estrous cyclicity, ovarian and uterine weights, ER $\alpha$ -immunoreactivity, olfactory  
458 preference, lordosis and anxiety-related behaviors, as well as cumulative locomotor activity. One-way  
459 ANOVA and Kruskal-Wallis tests were used to assess the same tests in Experiment 2. Paired t-test and  
460 Wilcoxon test were used in both experiments to analyze partner preference in three-chamber and  
461 urinary preference tests. Two-way ANOVA was used in both experiments to assess the effect of

462 treatment on body weight, number of entries in the stimulus arms for olfactory preference and 2-hour  
463 monitoring of locomotor activity.

464

#### 465 **Ethical approval**

466 The study was conducted according to the European legal requirements and received the approval of  
467 the «Charles Darwin» Ethical committee (project number 32459). The experiments were in compliance  
468 with the ARRIVE guidelines.

469

#### 470 **Data availability**

471 The authors declare that the data supporting the findings of this study are available within the paper  
472 and its supplementary information files.

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549

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552 supported by the Agence Nationale de la Recherche (ReproFun, 2020) in France (to CG and SMK).

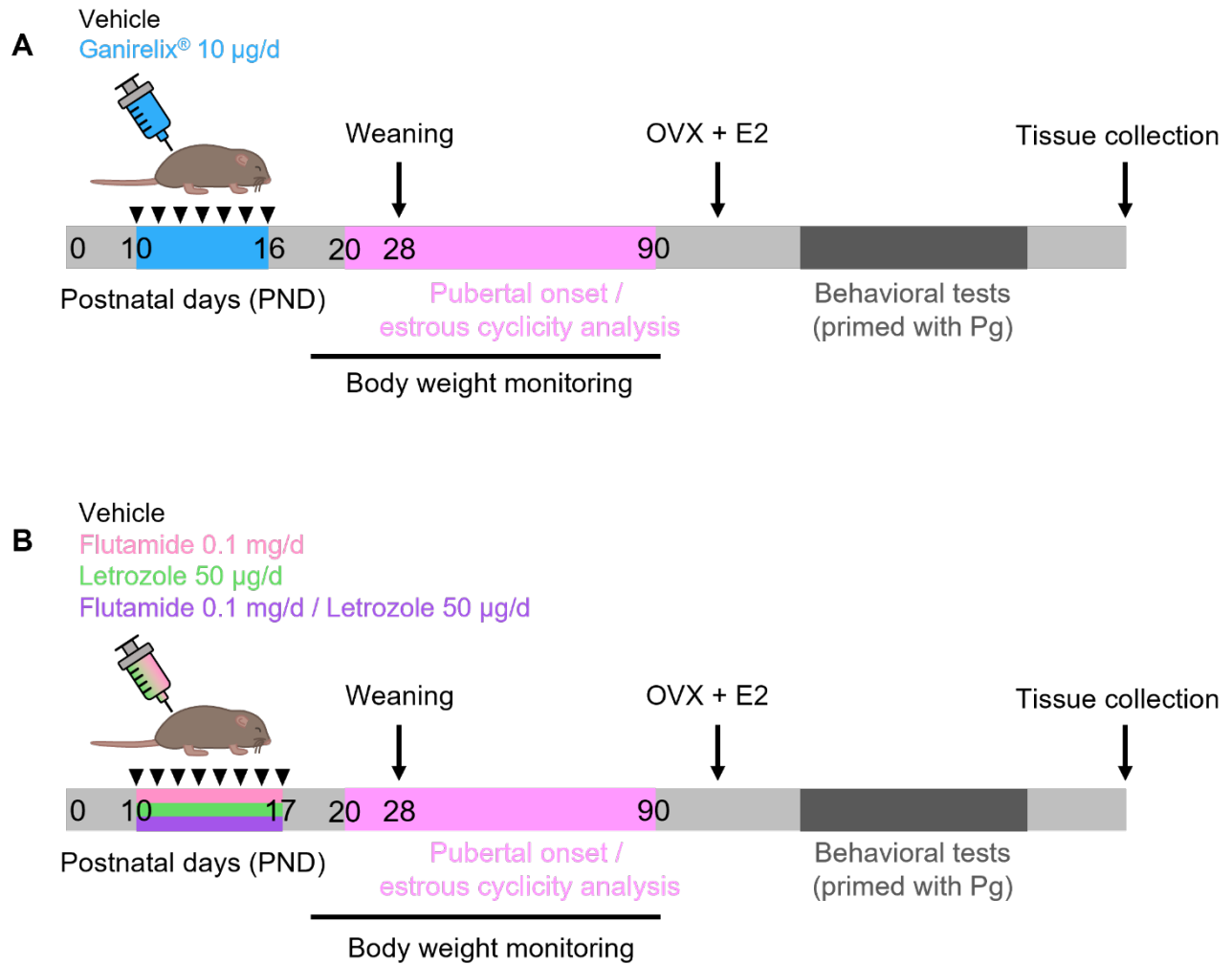
553 **Author's contribution**

554 CG, SMK and LN designed the study. TT, CP and LN carried out the experiments. TT, SMK and LN  
555 analyzed the data. TT, SMK and LN wrote the manuscript. All the authors corrected and edited the  
556 manuscript.

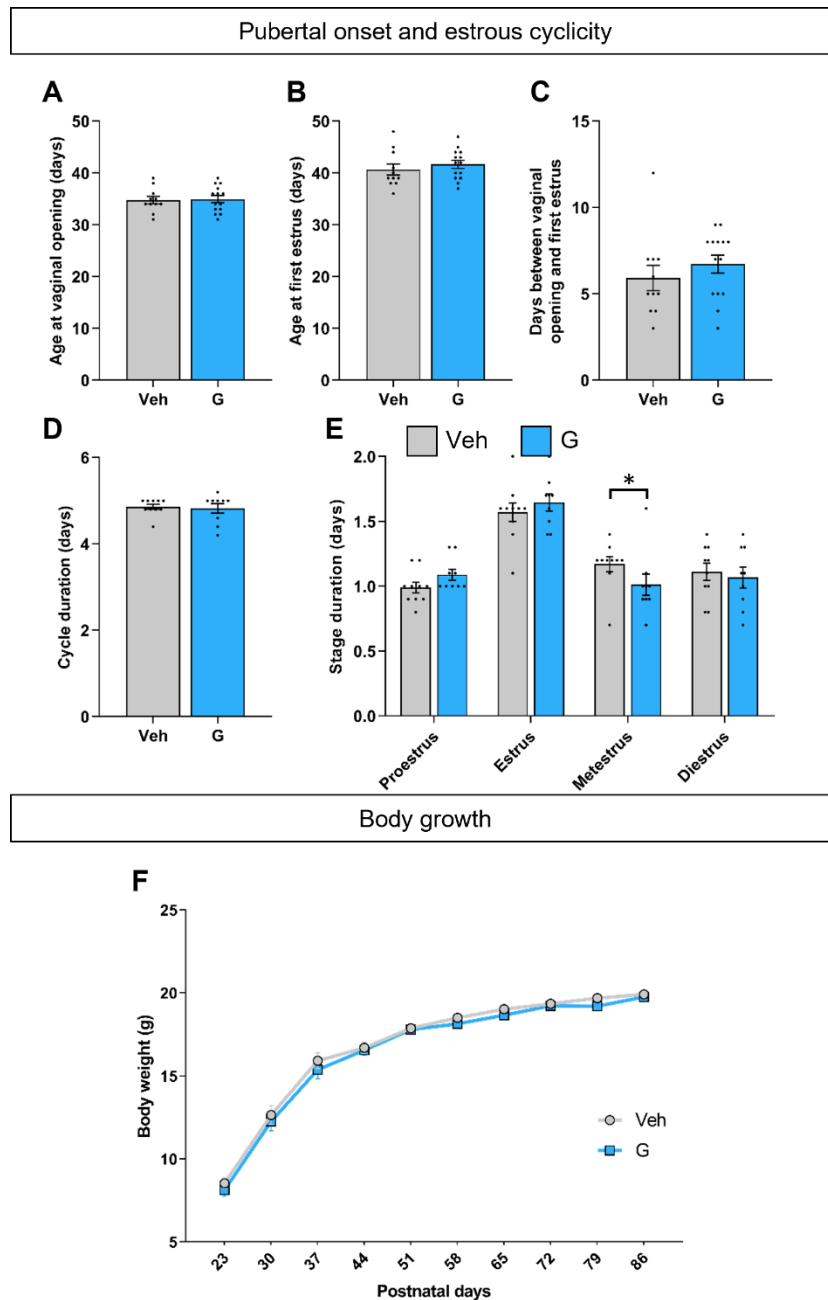
557 **Competing interests**

558 The authors declare no competing interests.

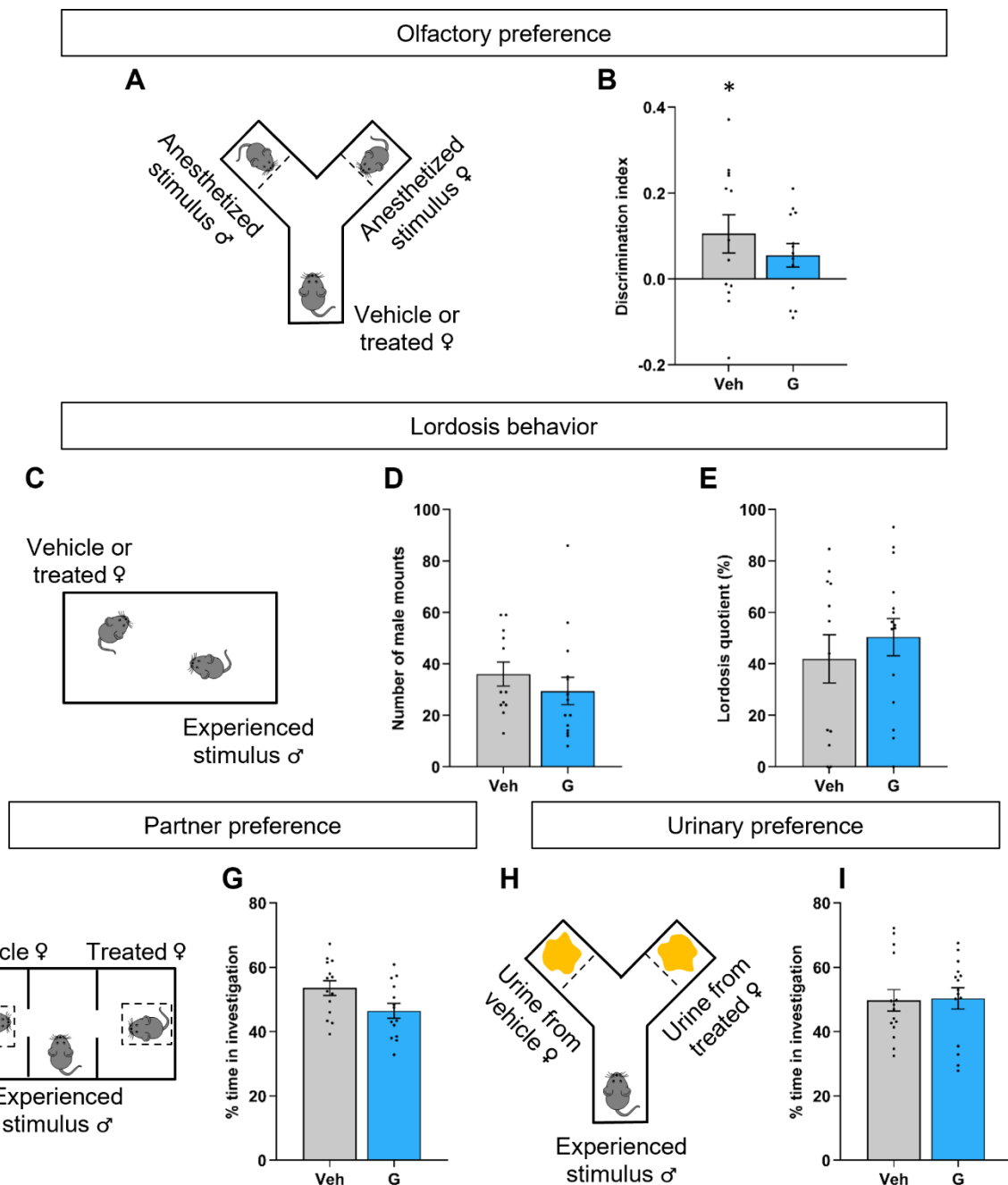
559 **Figures**



560 **Figure 1. Schematic representation of the experimental design.** **A.** In Experiment 1, female mice  
 561 were daily treated with the vehicle or Ganirelix® at the indicated doses from postnatal day 10 (PND10)  
 562 to PND16. **B.** In Experiment 2, female mice were daily treated with the vehicle, flutamide (F), letrozole  
 563 (L) or both (F/L) at the indicated doses from PND10 to PND17. For both experiments, body weight,  
 564 pubertal onset and estrous cyclicity were monitored in intact females. Females were then  
 565 ovariectomized and received estradiol implants (OVX+E2) and were primed with progesterone (Pg)  
 566 before each behavioral test. At the end of behavioral tests, females were euthanized and tissues were  
 567 collected.



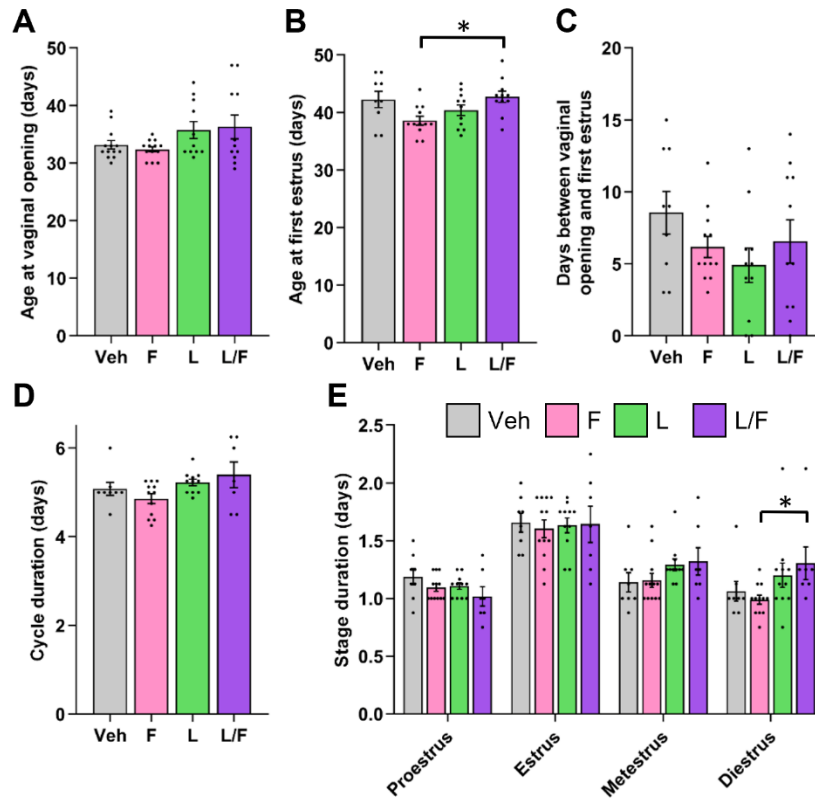
568 **Figure 2. Effects of minipubertal treatment with Ganirelix<sup>®</sup> on pubertal onset, estrous cyclicity**  
 569 **and body growth.** A-C. Female mice treated with the vehicle (Veh) or Ganirelix (G) from postnatal  
 570 (PND)10 to PND16 were analyzed for the age at vaginal opening (A) and first estrus (B) as well as the  
 571 interval between these two pubertal markers (C). D-E. The estrous cycle was monitored in adult control  
 572 and Ganirelix-treated females. The duration of the estrous cycle (D) and of each stage (E) are indicated;  
 573 (\*p < 0.05 for the metestrus phase). F. Body weight was followed every week from PND20 to PND86  
 574 for each female. Data are presented as means ± S.E.M. (n = 9-15 per treatment).



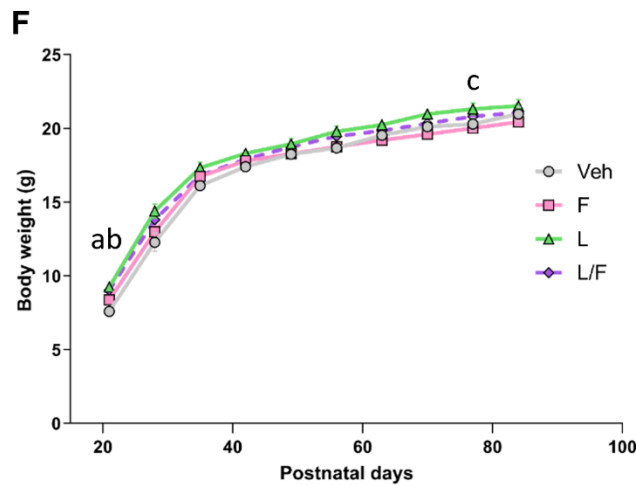
575 **Figure 3. Effects of minipubertal treatment with Ganirelix® on female sexual behavior. A-B.** In  
 576 the olfactory preference test, female mice treated with the vehicle (Veh) or Ganirelix (G) had choice  
 577 to chemoinvestigate anesthetized male or female stimuli (A). The discrimination index, time spent in  
 578 male investigation minus the time spent in female investigation divided by the total time of  
 579 investigation is indicated (B). \*p < 0.05, positive index for the control group. C-E. Lordosis behavior  
 580 was tested for sexually-experienced females in the presence of sexually experienced males for 30 min

581 (C). The number of male mounts (D) and lordosis quotient (E) are indicated. F-G. In the three-  
582 chambered test, sexually experienced males had choice to investigate Veh- or G-treated females (F).  
583 The percentage of time spent investigating each female is indicated (G). H-I. In the urinary preference  
584 test, sexually experienced males had choice to investigate urine from Veh- or G-treated females (H).  
585 The percentage of time spent investigating each urine stimulus is indicated (I). Data are presented as  
586 means  $\pm$  S.E.M. (n = 12-15 per treatment).

Pubertal onset and estrous cyclicity

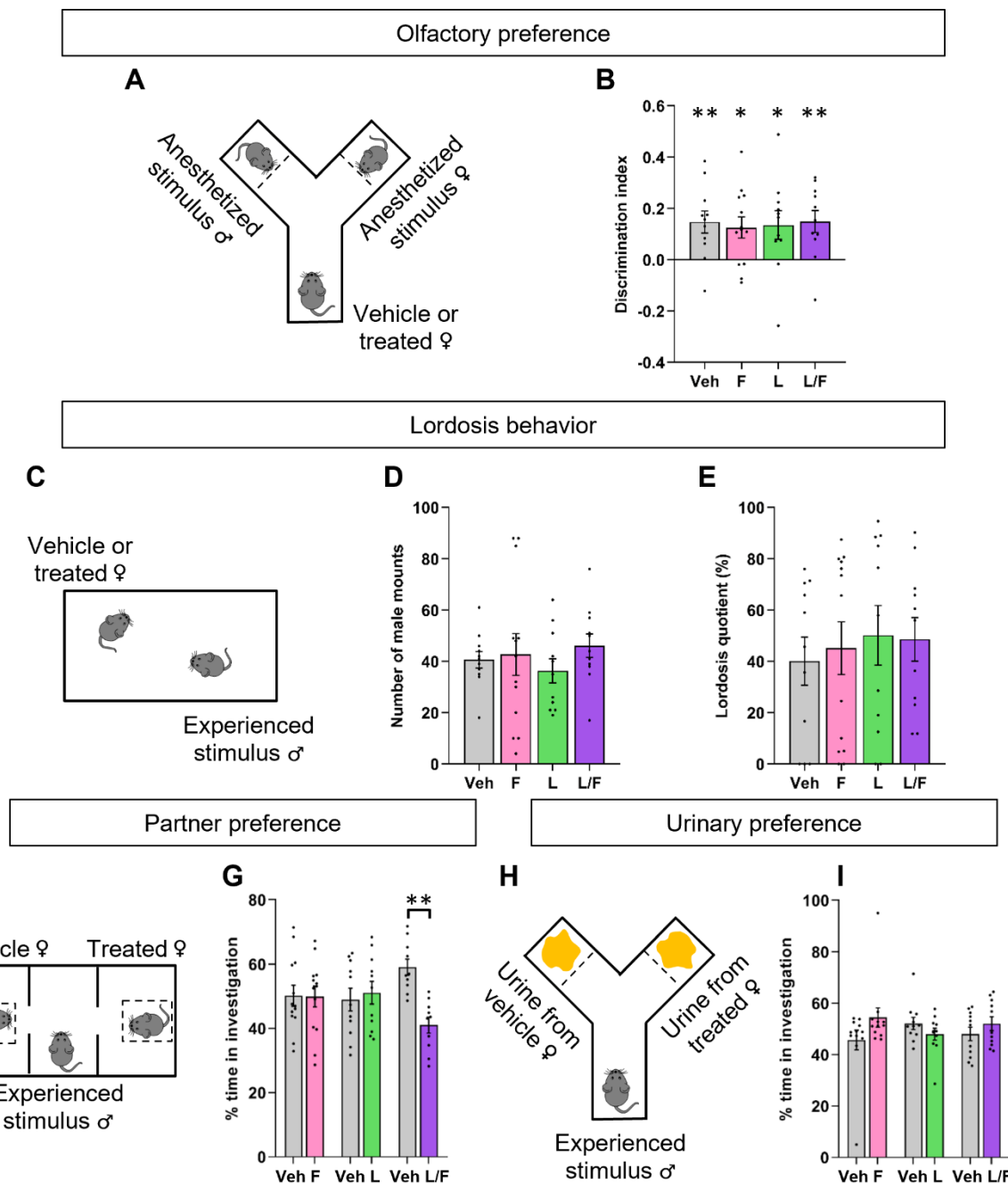


Body growth



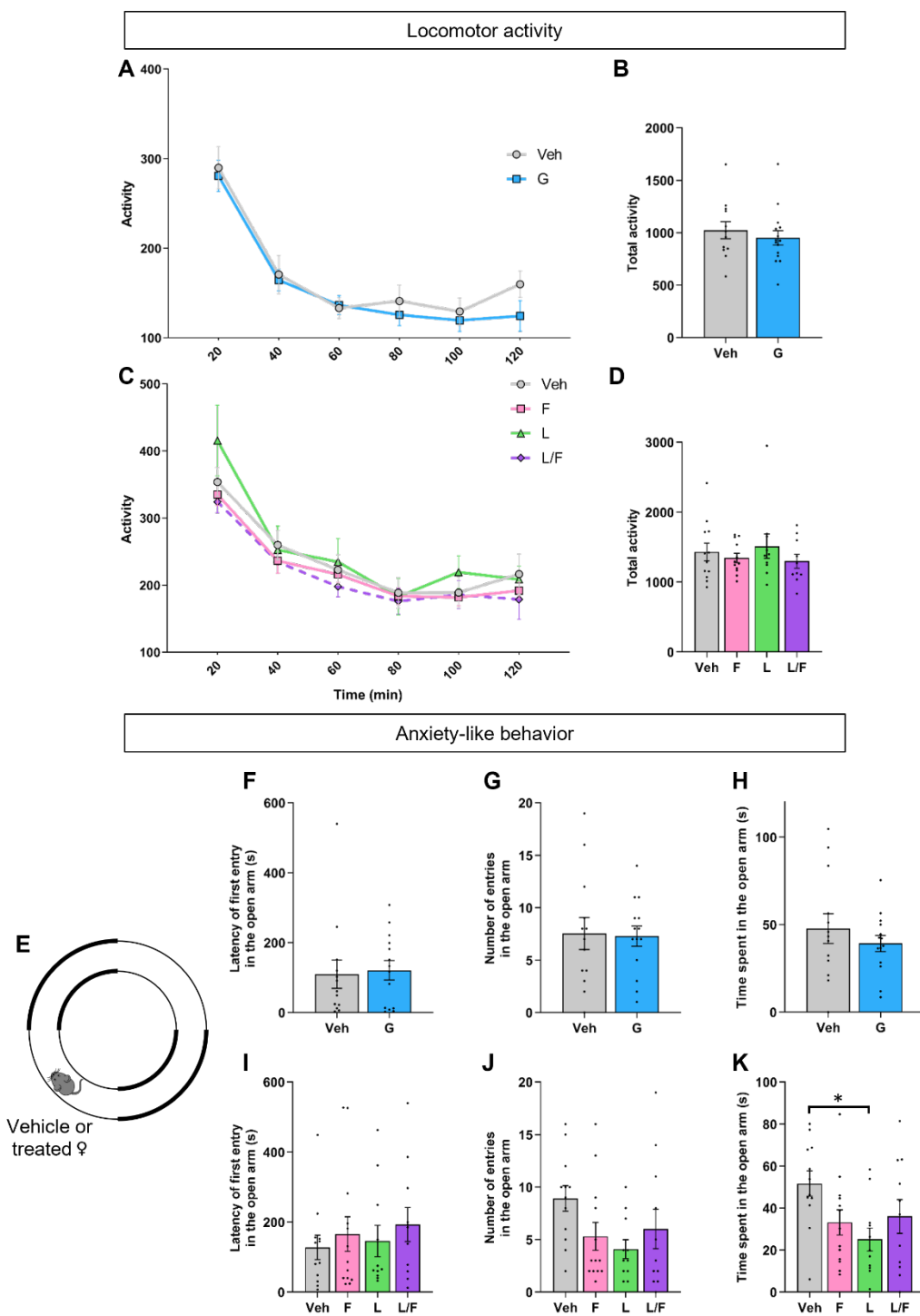
587 **Figure 4. Effects of separate or combined treatment with flutamide and letrozole during**  
 588 **minipuberty on pubertal onset, estrous cyclicity and body growth. A-C.** Female mice treated with  
 589 the vehicle (Veh), flutamide (F), letrozole (L) or both (F/L) from postnatal day (PND)10 to PND17  
 590 were analyzed for the age at vaginal opening (A), age at first estrus (B) and interval between the two  
 591 pubertal markers (C). \* $p < 0.05$  for the indicated groups. D-E. Duration of the estrous cycle (D) and of

592 each stage (E) in adult females. F. Body weight was monitored weekly from PND21 to PND86, <sup>a</sup>p  
593 <0.05 between Veh and L, <sup>b</sup>p < 0.05 between Veh and F/L, <sup>c</sup>p < 0.05 between F and L). Data are  
594 presented as means ± S.E.M. (n = 8-13 per treatment).



595 **Figure 5. Effects of separate and combined treatment with flutamide and letrozole during**  
 596 **minipuberty on female sexual behavior. A-B.** In the olfactory preference test, female mice treated  
 597 with the vehicle (Veh), flutamide (F), letrozole (L) or both (F/L) had choice to chemoinvestigate  
 598 anesthetized male or female stimuli (A). The discrimination index, time spent by exposed females in  
 599 male investigation minus the time spent in female investigation divided by the total time of  
 600 investigation (B). \* $p < 0.05$ , positive index for the four group. C-E. Lordosis behavior was tested for

601 sexually-experienced females in the presence of sexually experienced males for 30 min (**C**). The  
602 number of male mounts (**D**) and lordosis quotient (**E**) are indicated. **F-G**. In the three-chamber test,  
603 sexually experienced males had choice to investigate either Veh- or F- (\*\*p < 0.01 for F/L) (**F**). The  
604 percentage of time spent investigating each female is indicated (**G**). **H-I**. In the urinary preference test,  
605 sexually experienced males had choice to investigate urine either from Veh- or F-, Veh- or L-, or Veh-  
606 or F/L-treated female (**H**). The percentage of time spent investigating each urine stimulus is indicated  
607 (**I**). Data are presented as means  $\pm$  S.E.M. (n = 10-13 per treatment).



608 **Figure 6. Effect of minipubertal treatment on locomotor activity and anxiety-like behavior. A-D.**  
 609 Locomotor activity was monitored in a circular corridor during 2 h. The activity monitored every 20  
 610 minutes (A, C) or cumulative activity (B, D) are presented for females treated with the vehicle (Veh)  
 611 and Ganirelix (G) groups in Experiment 1, and flutamide (F) alone, letrozole alone or both flutamide  
 612 and letrozole (F/L) in Experiment 2. E-K. Anxiety-like behavior was assessed in a O-maze during 9

613 min (**E**). The latency to the first entry into the open arm (**F, I**), the number of entries into the open arm  
614 (**G, J**) and the time spent into the open arm (**H, K**) are indicated. Data are presented as means  $\pm$  S.E.M.  
615 (n = 12-15 per treatment for Experiment 1; n = 10-13 per treatment for Experiment 2) \*p < 0.05 for the  
616 indicated groups.

1 Effects of mini-puberty disruption on the expression of sexual behavior in female mice

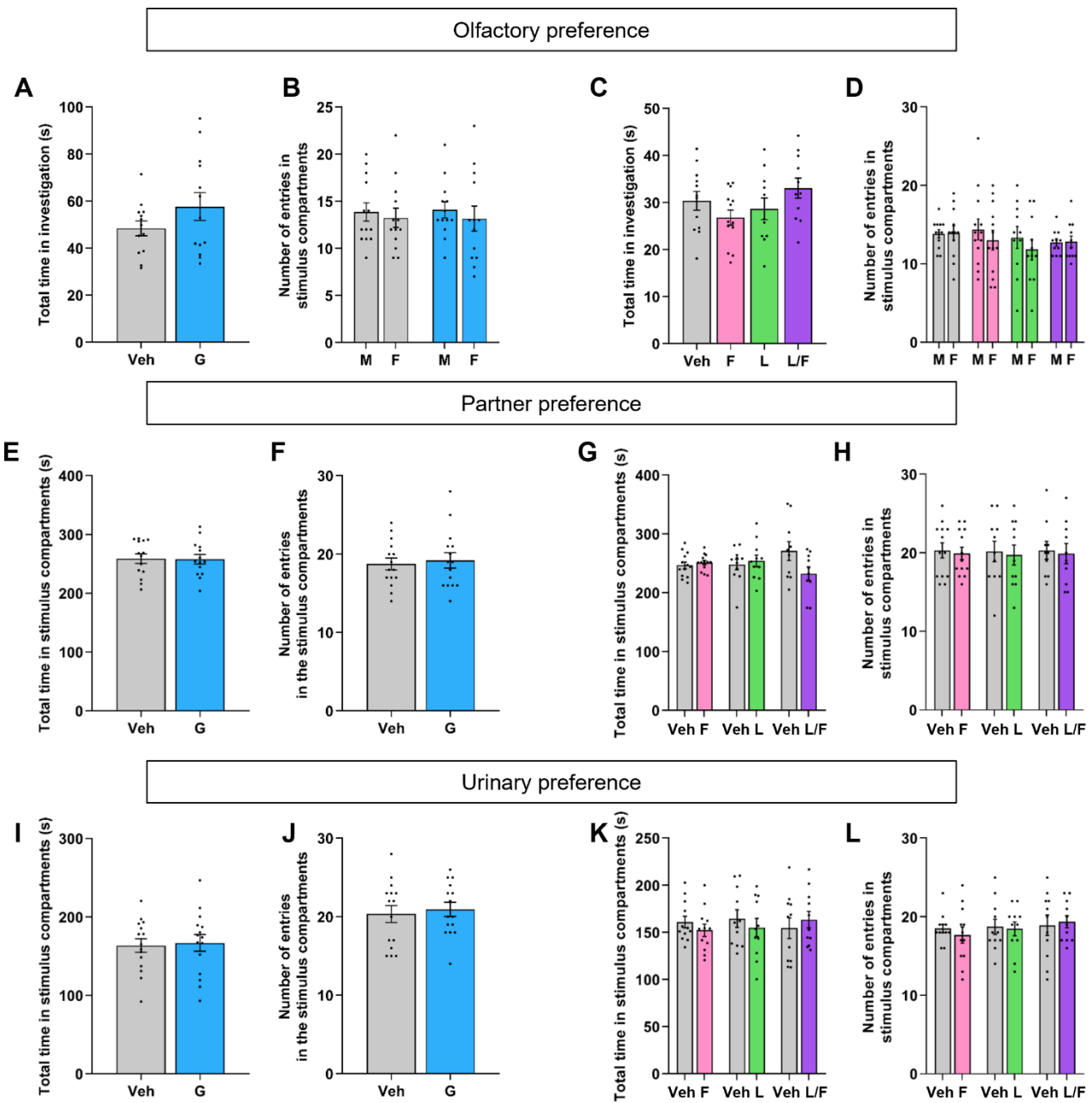
2 Thomas Torres, Caroline Parmentier, Céline Guigon, Sakina Mhaouty-Kodja, Lydie Naulé

3

4 Figure S1

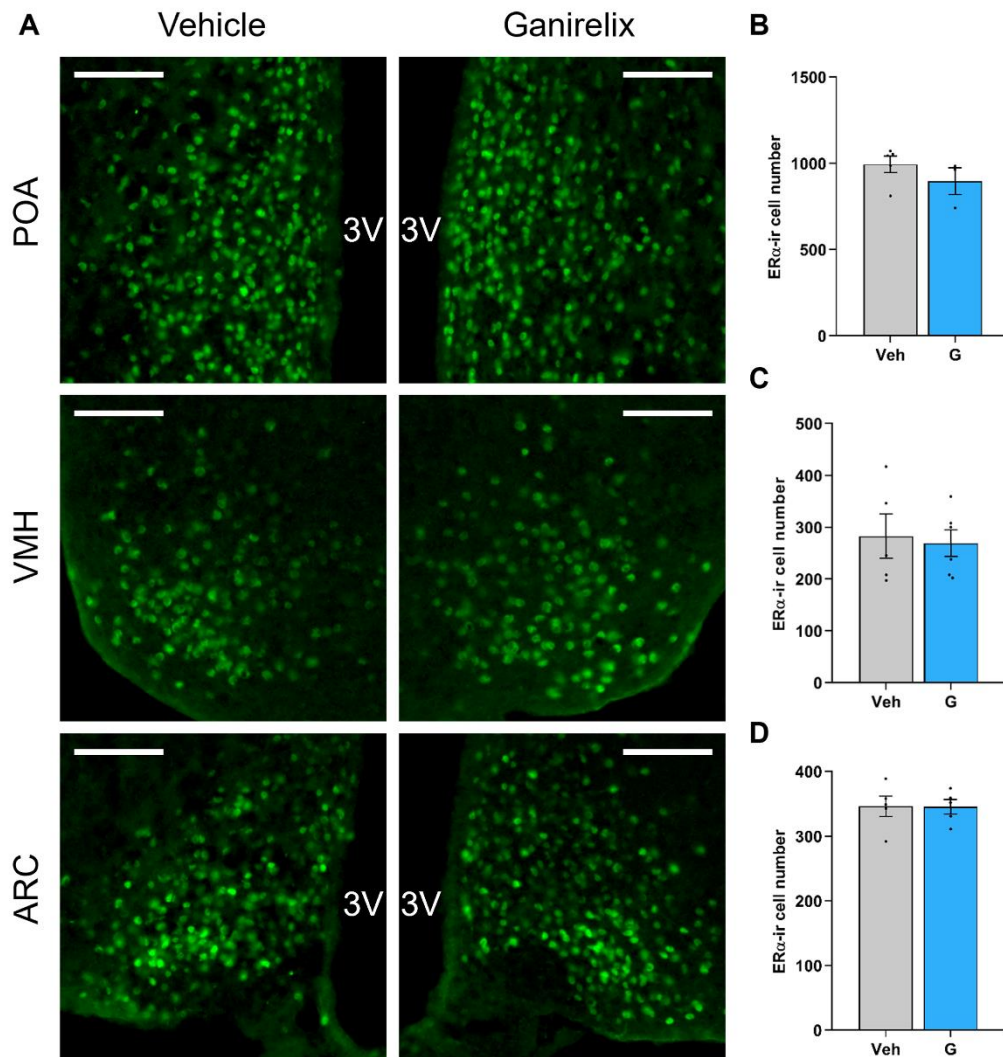
5 Figure S2

6 Figure S3



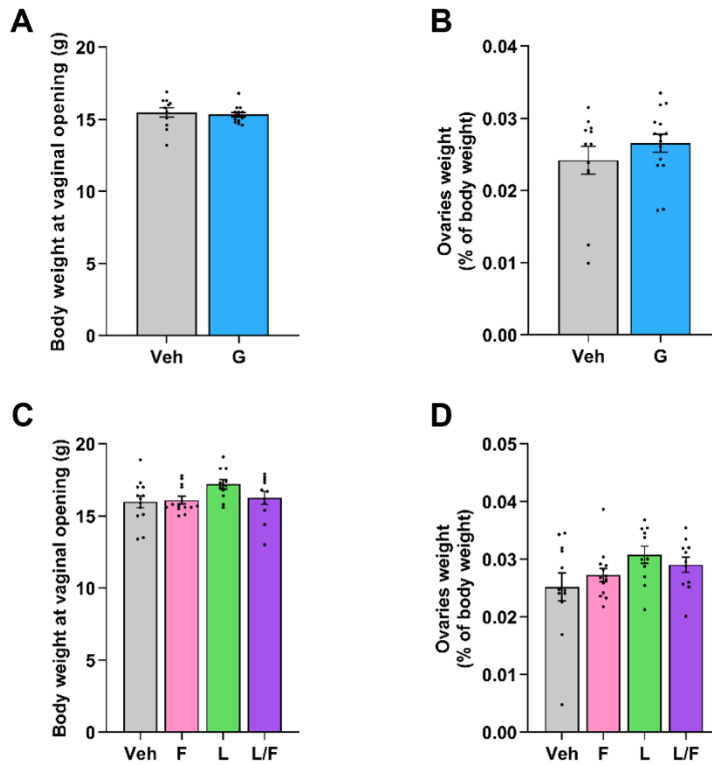
7 **Figure S1. Effect of minipubertal treatment on female sexual behavior.** A-D. In the olfactory  
8 preference test, female mice treated with vehicle (Veh), Ganirelix (G), flutamide (F), letrozole (L) or  
9 flutamide/letrozole (F/L) had choice to chemoinvestigate anesthetized male or female stimuli. The total  
10 time in investigation (A, C) and the number of entries in both stimulus compartment (B, D) were  
11 assessed. In the three-chambered tests, sexually experienced males had choice to investigate either  
12 Veh- or G-treated females (E, F) or either from Veh- or F-, Veh- or L-, or Veh- or F/L-treated females  
13 (G, H). The total time males spent in stimulus compartments (E, G) and the number of entries in

14 stimulus compartments is indicated (**F-H**). In the urinary preference tests, sexually experienced males  
15 had choice to investigate urine from either Veh- or G-treated females (**I, J**) or either from Veh- or F-,  
16 Veh- or L-, or Veh- or F/L-treated females (**K, L**). The total time males spent in stimulus compartments  
17 (**I, K**) and the number of entries in stimulus compartments is indicated (**J-L**). Data are presented as  
18 means  $\pm$  S.E.M. (n = 12-15 per treatment group for Experiment 1; n = 10-13 per treatment group for  
19 Experiment 2).



20 **Figure S2. Effect of minipubertal treatment with Ganirelix<sup>®</sup> on ER $\alpha$ -immunoreactivity in the**  
 21 **neural substrates of sexual behavior. A.** Representative nuclear ER $\alpha$ -immunolabeling in the POA,  
 22 VMH and ARC of vehicle- (Veh) and Ganirelix-treated female (G). Scale bar = 100 $\mu$ m. **B-D.**  
 23 Quantitative analyses of the number of ER $\alpha$ -immunoreactive cells in the POA (**B**), VMH (**C**) and ARC  
 24 (**D**). Data are presented as means  $\pm$  S.E.M. (n = 3-6 per treatment).

Physiological parameters



25 **Figure S3. Effect of minipubertal treatment on physiological parameters. A, C.** The body weight  
 26 at vaginal opening is indicated for Experiment 1 (A) and Experiment 2 (C). **B, D.** 2 weeks before sexual  
 27 behavior tests, females treated with vehicle (Veh), Ganirelix (G), flutamide (F), letrozole (L) or  
 28 flutamide/letrozole (F/L) were ovariectomized and the ovaries were weighted for Experiment 1 (B)  
 29 and Experiment 2 (D). Data are presented as means  $\pm$  S.E.M. (n = 12-15 per treatment for Experiment  
 30 1; n = 10-13 per treatment for Experiment 2).