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1 Male minipuberty involves the gonad-independent activation of preoptic nNOS neurons

2
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16 17 Abstract

18
19 **Background.** The maturation of the hypothalamic-pituitary-gonadal (HPG) axis is crucial for
20 the establishment of reproductive function. In female mice, neuronal nitric oxide synthase (nNOS)
21 activity appears to be key for the first postnatal activation of the neural network promoting the release
22 of gonadotropin-releasing hormone (GnRH), i.e. minipuberty. However, in males, the profile of
23 minipuberty as well as the role of nNOS-expressing neurons remain unexplored.

24 **Methods.** nNOS-deficient and wild-type mice were studied during postnatal development.
25 The expression of androgen (AR) and estrogen receptor alpha (ER α) as well as nNOS
26 phosphorylation were evaluated by immunohistochemistry in nNOS neurons in the median preoptic
27 nucleus (MePO), where most GnRH neuronal cell bodies reside, and the hormonal profile of nNOS-
28 deficient male mice was assessed using previously established radioimmunoassay and ELISA
29 methods. Gonadectomy and pharmacological manipulation of ER α were used to elucidate the
30 mechanism of minipubertal nNOS activation and the maturation of the HPG axis.

31 **Results.** In male mice, minipubertal FSH release occurred at P23, preceding the LH surge at
32 P30, when balanopreputial separation occurs. Progesterone and testosterone remained low during
33 minipuberty, increasing around puberty, whereas estrogen levels were high throughout postnatal
34 development. nNOS neurons showed a sharp increase in Ser¹⁴¹² phosphorylation of nNOS at P23, a
35 phenomenon that occurred even in the absence of the gonads. In male mice, nNOS neurons did not
36 appear to express AR, but abundantly expressed ER α throughout postnatal development. Selective

37 pharmacological blockade of ER α during the infantile period blunted Ser¹⁴¹² phosphorylation of
38 nNOS at P23.

39 **Conclusions.** Our results show that the timing of minipuberty differs in male mice when
40 compared to females, but as in the latter, nNOS activity in the preoptic region plays a role in this
41 process. Additionally, akin to male non-human primates, the profile of minipuberty in male mice is
42 shaped by sex-independent mechanisms, and possibly involves extragonadal estrogen sources.

43

44 Keywords: minipuberty; neuronal nitric oxide synthase (nNOS); gonadotropin-releasing hormone
45 (GnRH); estrogen; hypothalamus

46

47 Introduction

48

49 Reproduction is orchestrated by the hypothalamus, and specifically neurons located in the
50 median preoptic nucleus (MePO) that synthesize gonadotropin-releasing hormone (GnRH) and
51 release it at their nerve terminals in the median eminence of the hypothalamus (Herbison, 2015;
52 Prevot, 2015; Moenter, 2017). From there, GnRH is carried by the pituitary portal circulation to the
53 anterior pituitary, where it stimulates the synthesis and secretion of the gonadotropins, luteinizing
54 hormone (LH) and follicle-stimulating hormone (FSH). The gonadotropins, as indicated by their
55 name, act on the gonads (i.e., the testes and ovaries) to control the production of sperm and eggs and
56 the secretion of sex steroids. The disruption in any of the above steps of the hypothalamic-pituitary-
57 gonadal (HPG) axis can have a detrimental impact on fertility. However, to elicit gonadotropin
58 release, coordinated GnRH neuronal activity that can promote meaningful episodes of GnRH
59 secretion is required. This neuronal activity is controlled by a dynamic array of internal and external
60 signals involving various neuropeptides, hormones and neurotransmitters, including gonadal steroids
61 themselves as well as the gaseous second messenger nitric oxide (NO) (Chachlaki et al., 2017a;
62 Chachlaki and Prevot, 2020; Delli et al., 2021).

63 The first postnatal activation of the HPG axis, which occurs during the infantile period in both
64 humans and mice, is attested to by a substantial release of FSH and LH (Kuiiri-Hänninen et al., 2014;
65 Prevot, 2015). Neuronal NO synthase (nNOS) neurons, which produce NO, are morphologically and
66 functionally associated with GnRH neuronal cell bodies and dendrites in the MePO and the organum
67 vasculosum of the lamina terminalis (OVLT or OV) (Chachlaki et al., 2017b). As such, they are
68 ideally positioned to play a role in the rapid integration and transmission of both gonadal and
69 metabolic signals to GnRH neurons, including during minipuberty. Increased nNOS activity – and
70 hence NO release – during minipuberty, by modulating the transcription, activity and release of
71 GnRH (Messina et al., 2016), could thus control the secretion of gonadotropins and subsequently, the
72 maturation and function of the neuroendocrine axis (Chachlaki et al., 2022).

73 In humans, gonadotropin profiles during minipuberty are sexually dimorphic. Minipuberty
74 induces an increase in the level of estradiol, promoting the maturation of ovarian follicles in girls,
75 whereas in boys, urinary levels of FSH are positively correlated with testicular growth, increasing the
76 level of circulating testosterone. The impairment of minipuberty may hence impede the development
77 of the reproductive system, affecting puberty and subsequently, adult fertility (Kuiri-Hänninen et al.,
78 2014). Interestingly, female nNOS-deficient mice demonstrate exacerbated minipuberty, with
79 aberrantly high FSH and LH levels during infantile life (Chachlaki et al., 2022), and estrogen plays a
80 crucial role in the minipubertal activation of nNOS neurons and the subsequent maturation of the
81 HPG axis (Chachlaki et al., 2017b; Chachlaki et al., 2022). However, not much is known about the
82 mechanisms at work during minipuberty in males.

83 Here, we explored whether the regulation of minipuberty was sex-dependent, and attempted
84 to determine the role played by NO signaling in the control of male minipuberty.

85

86 Materials and Methods

87

88 ANIMALS

89 All C57Bl/6J mice were housed under specific pathogen-free conditions in a temperature-
90 controlled room (21-22°C) with a 12h light/dark cycle and ad libitum access to food and water.
91 Experiments were performed on male and female C57Bl/6J mice (Charles River Laboratories), Nos1-
92 deficient (Nos1^{-/-}, B6.129S4-Nos1^{tm1Plh/J}) mice (26) and Gnrh::Gfp mice (a generous gift of Dr.
93 Daniel J. Spergel, Section of Endocrinology, Department of Medicine, University of Chicago, IL)
94 (97). Nos1^{-/-}; Gnrh::Gfp mice were generated in our animal facility by crossing Nos1^{-/+} mice with
95 Gnrh::Gfp mice. Animal studies were approved by the Institutional Ethics Committees for the Care
96 and Use of Experimental Animals of the Universities of Lille; all experiments were performed in
97 accordance with the guidelines for animal use specified by the European Union Council Directive of
98 September 22, 2010 (2010/63/EU) and were approved by the French Department of Research
99 (APAFIS#2617-2015110517317420v5).

100

101 ORCHIDECTOMY

102 Two independent litters of Gnrh::Gfp mice were used for orchidectomies. At postnatal day 10
103 (P10) male littermates were either orchidectomized (ORX), or just laparotomized (Sham), under
104 general anaesthesia (induction 4% in air 2 L/min, then 1.5% in air 0.3 L/min), after local injection of
105 lidocaine (30 microliters of a 0.5% solution, s.c.) and preemptive Meloxicam treatment (5 mg/kg).

106

107 MPP TREATMENT

108 Gnrh::Gfp pups from three independent litters received a daily intraperitoneal injection of
109 MPP dihydrochloride (1,3-Bis(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyloxy)phenol]-1H-

110 pyrazole dihydrochloride hydrate, Methyl-piperidino-pyrazole hydrate; 0.1mg/kg; dissolved at 10mM
 111 in DMSO/ saline mixture), a high selective antagonist of estrogen receptor α (Tocris, Cat# 1991), or
 112 vehicle (DMSO/ saline). Treatment started at postnatal day 10 and terminated at postnatal day 23
 113 (lights on). Upon completion of the treatment blood was collected (lights off) and mice were perfused
 114 and processed for IHC analysis of brain sections.

115

116 QUANTITATIVE RT-PCR ANALYSES

117 For transcriptomic analysis all animals used were sacrificed by decapitation at the postnatal
 118 day indicated. The preoptic area of the hypothalamus (POA), the pituitary gland and the testis were
 119 collected in an Eppendorf tube and snap frozen in liquid nitrogen (or post-fixed for histological
 120 analysis, see below). The POA and the pituitary gland were dissociated in 40 μ l of extraction buffer
 121 [0.1% Triton® X-100 (Sigma-Aldrich) and 0.4 U/ μ l RNaseOUT™ (Life Technologies Invitrogen,
 122 Cat# 10777019)] and the testicle was processed for RNA extraction using the E.Z.N.A.® Total RNA
 123 Kit II (Omega Bio-Tek, Cat# R6934-02, Lot# R693425535-3), following the manufacturers'
 124 protocol. The samples were reverse transcribed using Transcriptase inverse MultiScribe™
 125 (Invitrogen, Cat# 4311235, Lot# 01105639). Real-time PCR was carried out on Applied Biosystems
 126 7900HT Fast Real-Time PCR System using exon-boundary-specific TaqMan® Gene Expression
 127 Assays (Applied Biosystems): Anti-Mullerian hormone (*Amh*-Mm00431795_g1), Androgen receptor
 128 (*Ar*-Mm00442688_m1), Cytochrome P450, family 19, subfamily a, polypeptide 1 (*Cyp19a1*-
 129 Mm00484049_m1), Cytochrome P450, family 17, subfamily a, polypeptide 1 (*Cyp17a1*-
 130 Mm00484040_m1), Estrogen receptor1 (alpha) (*Esr1*-Mm00433149_m1), Estrogen receptor 2 (beta)
 131 (*Esr2*-Mm00599821_m1), Follicle stimulating hormone beta (*Fshb*-Mm00433361_m1), Follicle
 132 stimulating hormone receptor (*Fshr*-Mm00442819_m1), Gonadotropin releasing hormone 1 (*Gnrh1*-
 133 Mm01315605_m1), Gonadotropin releasing hormone receptor (*Gnrhr*-Mm00439143_m1),
 134 Hydroxysteroid (17-beta) dehydrogenase 3 (*Hsd17b3*-Mm00515131_m1), 3 beta- and steroid delta-
 135 isomerase 6 (*Hsd3b6*-Mm07306505_m1), Inhibin alpha (*Inha*-Mm00439683_m1), Inhibin beta
 136 (*Inhbb*- Mm03023992_m1), Insulin-like 3 (*Insl3*-Mm01340353_m1), Kiss-1 metastasis-suppressor
 137 (Mm03058560_m1), Kiss1 receptor (*Kiss1r*-Mm00475046_m1), Luteinizing hormone beta (*Lhb*-
 138 Mm00656868_g1), Luteinizing hormone receptor (*Lhcgr*-Mm00442931_m1), Nitric oxide synthase 1,
 139 neuronal (*Nos1*- Mm01208059_m1), Progesterone receptor (*Pgr*-Mm00435628_m1). Control
 140 housekeeping genes: Eukaryotic 18S rRNA (*Rn18S*-Mm03928990_g1), Actin beta (*Actb*-
 141 Mm00607939_s1).

142

143 ANALYSIS OF MALE GONADS

144 After surgical removal, testis was dried on filter paper and weighted. Testicular weight was
 145 eventually normalized over total body weight of the animal prior to sacrifice. For histological

146 preparations, testis was fixed in 4% PFA overnight. Tissue was then progressively dehydrated with
147 ethanol/xylene baths, before being paraffin-embedded. Testis were cut in 6µm sections with a
148 microtome (Leica, RM2245) and stained with hematoxylin and eosin. Sections were eventually
149 digitalized using Zeiss Axioscan Z1 with a 20×/0.8 objective in brightfield. For each animal, the
150 number of seminiferi tubules and the cross-section area were manually counted using ZEN (ZEISS).
151 The quantification was realized in the whole testis every 50µm, excluding the extremities of the organ
152 (150 µm).

153

154 IMMUNOHISTOCHEMISTRY

155 *Tissue Preparation:* Mice were anesthetized with a lethal dose of pentobarbital (DOLÉTHAL®,
156 5mg/kg) and locally treated with Lidocain (30 µl of a 0.5% solution). Animals were perfused
157 thoroughly with cold saline, until exsanguination, then perfused with cold fixative solution [4%
158 paraformaldehyde (PFA), in 0.1M PB, pH 7.4]. The brain was extracted and post-fixed with the same
159 fixative solution for 2h at 4°C, then cryoprotected in PB 0.1M 20% sucrose for 24h at 4°C.
160 Afterwards, the brain was embedded in OCT medium (Tissue-Tek), frozen on dry ice, and stored at
161 -80°C until sectioning. Tissues were cryosectioned (Leica cryostat) coronally at 35 µm (free-floating
162 sections).

163 *Antibodies:* The goat polyclonal anti-nNOS antibody (1:1000; Cat# OSN00004G, Lot#VF3001511)
164 and the rabbit polyclonal anti-Ser1412 phospho-nNOS antibody (1:500; Cat# PA1-032, Lot#
165 UB274796) were purchased from ThermoScientific. The rat polyclonal anti-GnRH (1:2500; Cat#
166 EH1044) was a generous gift from Professor Hrabovszky (Laboratory of Reproductive Neurobiology,
167 Institute of Experimental Medicine, Budapest, Hungary). The rabbit polyclonal anti-estrogen receptor
168 α (1:500; Cat# 06-935, Lot#3701815) and anti-androgen receptor (1:1000; Cat# 06-680, Lot#
169 38113557) were purchased from Millipore. The Alexa Fluor 647-conjugated donkey anti-goat
170 secondary antibody (1:500; Cat# A21447, Lot# 2465096) used for nNOS immunolabeling, the Alexa
171 Fluor 568-conjugated donkey anti-rabbit secondary antibody (1:500; Cat# A10042, Lot# 2306809)
172 used for phospho-nNOS, androgen receptor and estrogen receptor α immunolabeling, and the Alexa
173 Fluor 488-conjugated donkey anti-rat secondary antibody (1:500; Cat# A21208, Lot# 2180272) used
174 for GnRH immunolabeling, were purchased from Invitrogen.

175 *Immunolabeling:* Sections were washed 3 times for 5 minutes each in PB 0.1M and then incubated in
176 blocking solution (5% NDS, 0.3% Triton X-100 in PB 0.1M) for 1 hour at room temperature. Sections
177 were incubated for 48-72 hours at 4°C with the primary antibodies diluted in blocking solution, then
178 rinsed 3 times for 5 minutes each in PB 0.1M. The sections were incubated with secondary antibodies
179 diluted in PB 0.1M for 1h at room temperature, then rinsed in PB 0.1M 3 times for 5 minutes each,
180 and finally incubated 7 minutes with DAPI (1:5000 in PB 0.1 M) for nuclear staining. After washing,
181 sections were mounted on glass slides and coverslipped under Mowiol medium.

182 *Digital image acquisition:* Images were acquired with a Zeiss Axio Imager Z2 microscope (Zeiss,
183 Germany). Alexa 488 was imaged using a 495 nm beam splitter with an excitation wavelength set at
184 450/490 nm and an emission wavelength set at 500/550 nm. Alexa 568 was imaged using a 570 nm
185 beam splitter with an excitation wavelength set at 538/562 nm and an emission wavelength set at
186 570/640 nm. Alexa 647 was imaged by using a 660 nm beam splitter with an excitation wavelength
187 set at 625/655 nm and an emission wavelength set at 665/715 nm. Nuclear staining (Hoechst) was
188 imaged by using a 395 nm beam splitter with an excitation wavelength set at 335/383 nm and an
189 emission wavelength set at 420/470 nm. Images were acquired with a Plan-Apochromat 20X
190 objective (numerical aperture NA = 0.80; thread type M27). Z-stack images were taken every 1 μm ,
191 for a total of 20 μm per sections. All the images are presented as maximal intensity projections (MIP)
192 of three-dimensional volumes along the optical axis. Images to be used for figures were adjusted for
193 brightness and contrast using Photoshop (Adobe Systems, San Jose, CA).

194 *Cell counting:* The nomenclature of brain regions used in this article corresponds to that described in
195 the Allen brain atlas (Lein et al., 2007). The quantification of nNOS neurons co-expressing estrogen
196 receptor α or androgen receptor was undertaken by counting the number of single-labeled nNOS-
197 positive neurons and double-labeled nNOS/estrogen receptor- α or nNOS/androgen receptor cells in
198 the OV, represented by plate 49 of the Allen Developing Mouse Brain Atlas. Cell counts were carried
199 out unilaterally, represented as percentage of nNOS neurons co-expressing estrogen receptor α or
200 androgen receptor, and the values from each mouse were averaged to determine mean counts and
201 SEM for each age group.

202

203 MEASUREMENT OF CIRCULATING HORMONES

204 *Radioimmunoassay (FSH):* Serum FSH measurement was carried out using a double antibody method
205 and the rat-FSH RIA kit supplied by the National Institutes of Health (Dr. A. F. Parlow, NIDDK-
206 NHPP, Torrance, CA). The primary antibody (NIDDK anti-rFSH-S-11, AB_2687903) was used at a
207 dilution factor of 1/31,250. Rat FSH antigen (NIDDK rFSH-I, AFP-5178B) was labeled with ^{125}I by
208 the chloramine-T method. The hormone concentration was calculated using the rat FSH reference
209 preparation (NIDDK rFSH-RP-2, AFP-4621B) as standard. The intra-assay and inter-assay
210 coefficients of variation were 7 % and 10 %. The sensitivity of the assay was 0.03 ng/100 μl . FSH
211 levels were measured using radioimmunoassay as previously described (Messina et al., 2016).

212 *Elisas (LH, 17 β -estradiol, Inhibin B, AMH, Testosterone, Progesterone):* Plasma LH was measured
213 using a highly sensitive Enzyme-linked Immunosorbent Assay (ELISA) as described elsewhere
214 (Steyn et al., 2013). The accuracy of hormone measurements was confirmed by the assessment of
215 rodent serum samples of known concentration (external controls). Serum 17 β -estradiol concentration
216 was determined by ELISA (Demeditec Diagnostics, Cat# DE2693) as described previously (Fonseca
217 et al., 2012). Inhibin B was measured using a commercial Enzyme-linked Immunosorbent Assay
218 (ELISA) multispecies kit (AnshLabs, Cat# AL-163), following the manufacturers' protocol. AMH

219 was measured using the commercial rat and mouse AMH Enzyme-linked Immunosorbent Assay
220 (ELISA) kit (AnshLabs, Cat# AL-113) as described elsewhere (Devillers et al., 2019). Plasmatic
221 concentrations of progesterone were assessed using a commercial kit (Demeditec Diagnostics, Cat#
222 DEV9988), following the manufacturers' protocol. Testosterone was measured using the commercial
223 rat/mouse ELISA kit (Demeditec Diagnostics, Cat# DEV9911).

224

225 STATISTICAL ANALYSIS

226 All analyses were performed using Prism 7 (Graphpad Software, San Diego, CA) and assessed for
227 normality (Shapiro–Wilk test) and variance, when appropriate. Sample sizes were chosen according to
228 standard practice in the field. The investigators were blinded to the group allocation during the
229 experiments. For each experiment, replicates are described in the figure legends. For animal studies,
230 data were compared using an unpaired two-tailed Student's t-test or a one-way ANOVA for multiple
231 comparisons against the control condition followed by Dunnett multiple comparison post-hoc test.
232 Data not following normal distribution were analyzed using either a Mann-Whitney U test
233 (comparison between two experimental groups) or Wilcoxon/Kruskal-Wallis test (comparison
234 between three or more experimental groups) followed by a Dunn's post hoc analysis. The number of
235 biologically independent experiments, sample size, P values, age and sex of the animals are all
236 indicated in the main text or figure legends. All experimental data are indicated as mean \pm s.e.m. The
237 significance level was set at $P < 0.05$. Symbols in figures correspond to the following significance
238 levels: ns. $P > 0.05$, * $P < 0.05$, ** $P < 0.001$, *** $P < 0.0001$.

239

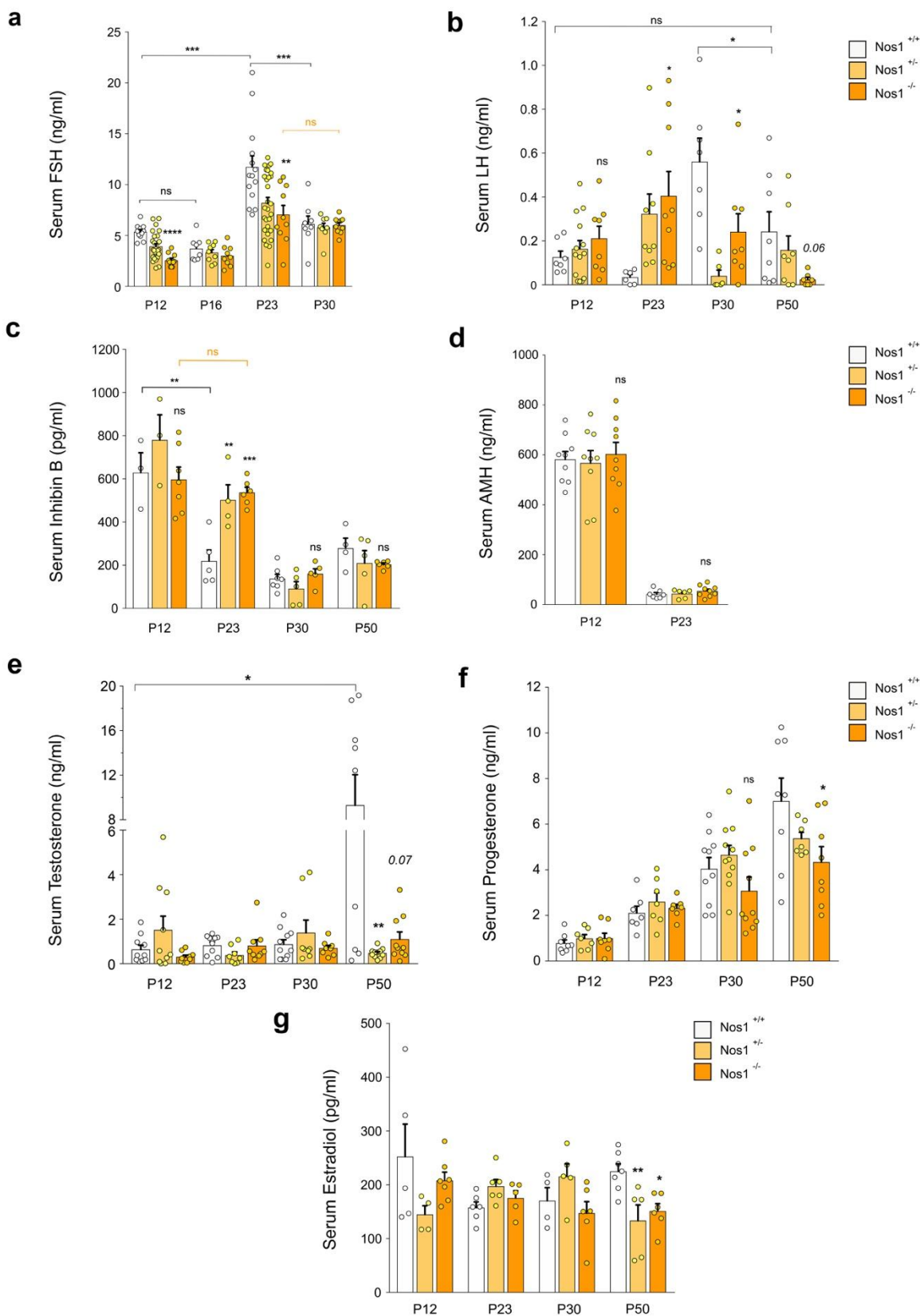
240 Results

241

242 **Minipubertal GnRH axis maturation differs in male and female mice**

243 In female mice, FSH and LH levels peak at around postnatal day (P) 12, returning to basal
244 levels after weaning, at around P23 (Prevot et al., 2003; François et al., 2017; Chachlaki et al., 2022).
245 In order to assess the timing of minipuberty in male mice, we analyzed gonadotropin levels, i.e. FSH
246 and LH, during different developmental stages: infantile (P12), juvenile (P23, P30) and peripubertal
247 (P50). Interestingly, in male mice, circulating FSH levels, reflecting *Gnrh* mRNA levels in the POA,
248 peaked at P23, i.e. at a time when minipuberty in females has terminated (Chachlaki et al., 2022),
249 returning to baseline by P30 (Fig. 1a, Suppl. Fig. 4). This minipubertal increase in *GnRH* production
250 does not seem to involve the kisspeptin pathway, since both *kiss1* and *kiss1r* mRNA levels remained
251 unchanged during this period (Suppl. Fig. 4). Surprisingly, however, serum LH levels in male mice
252 remained very low throughout the infantile and early juvenile period, but increased at P30, a week
253 later than FSH and around the time that balanopreputial separation normally occurs, returning to basal
254 values at P50 (Fig. 1b). Minipuberty thus seems to be a biphasic period rather than a single time point
255 or event in males.

256 To better understand the interplay between the maturational processes taking place both in the
257 hypothalamus and along the HPG axis of male mice, we measured circulating levels of the sex steroid
258 hormones (estradiol, progesterone and testosterone) as well as inhibin B and AMH during postnatal
259 development. Circulating testosterone levels in male mice remained low until the juvenile stage,
260 abruptly increased peripubertally (P50) (Fig. 1e). In contrast, serum progesterone levels steadily
261 increased over the period studied (Fig. 1f), while serum estradiol levels were consistently elevated
262 during postnatal development, with no marked difference between the various stages studied (Fig.
263 1g). Circulating AMH levels revealed relatively high levels in the serum of P12 male mice, but
264 dropped by almost 95% by the time minipuberty began at P23 (Fig. 1d). Similarly, inhibin B levels
265 decreased by more than half between P12 and P23, when FSH levels peaked in male wild-type mice
266 (Fig. 1c).



267

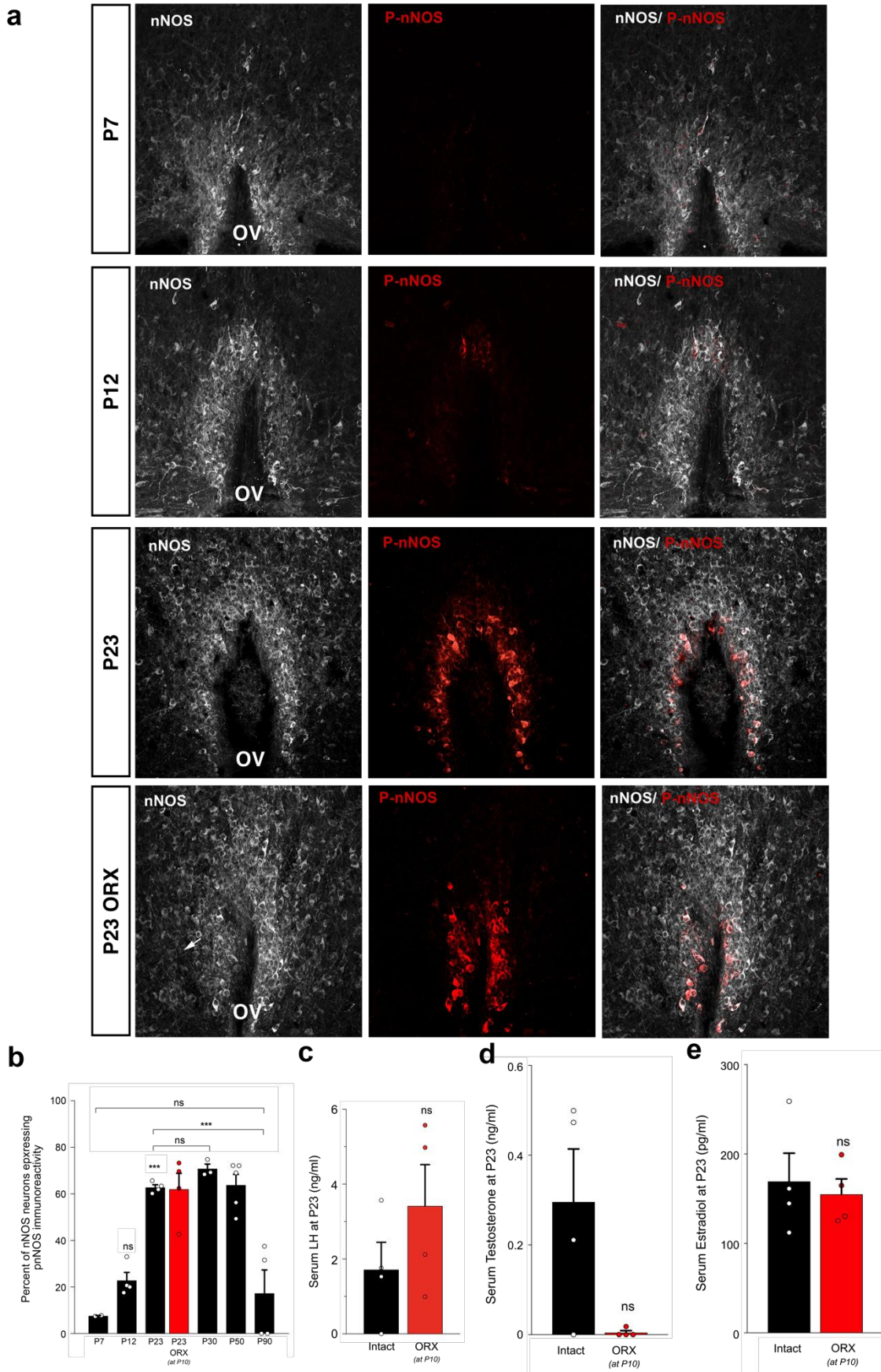
268 **Figure 1. nNOS activity shapes the pattern of minipuberty in male mice and alters the pubertal profile of**269 **circulating sex hormones. (a-g) Circulating levels of (a) FSH, (b) LH, (c) inhibin B, (d) AMH, (e) testosterone,**270 **(f) progesterone and (g) estradiol in male *Nos1*^{+/+}, *Nos1*^{+/-} and *Nos1*^{-/-} mice during different postnatal stages**271 **of development. Hormonal values in *Nos1*^{+/+} mice are compared with those of *Nos1*^{+/-} and *Nos1*^{-/-} mice for**

272 each group of measurements (one-way ANOVA with Tukey's post-hoc test for a-d, f-g and Kruskal-Wallis with
273 Dunn's post-hoc test for e). When applicable, comparisons between two developmental stages of the same
274 genotype are indicated in the graph (unpaired t-test for a-d, f-g and Mann-Whitney U test for e). * P < 0.05; ** P
275 < 0.01; *** P < 0.001. Values indicate means \pm SEM. N=3-8 independent litters.

276

277 **nNOS activity increases during minipuberty in the male hypothalamus**

278 The infantile period has been recognized as key for the establishment of a mature GnRH
279 neural network, including the activation of nNOS neurons in the OV/MePO in female mice (Messina
280 et al., 2016; Chachlaki et al., 2017a; Pellegrino et al., 2021; Chachlaki et al., 2022). In order to study
281 this preoptic nNOS neuronal population in males during sexual maturation, we measured
282 immunoreactivity for nNOS as well as phosphorylation-activated nNOS (P-nNOS), used as a
283 surrogate marker of nNOS activity (Rameau et al., 2007; Parkash et al., 2010), in the male OV during
284 several developmental stages: neonatal (P7), infantile (P12), juvenile (P23, P30), peripubertal (P50)
285 and adult (P90). Our results show that nNOS immunoreactivity was already present in the OV/MePO
286 by the neonatal period, and nNOS mRNA and protein expression not change substantially during
287 postnatal development (Fig. 2a-b, S1, 4). Phosphorylation of the nNOS protein in the male mouse
288 hypothalamus was negligible during the neonatal period (Fig. 2a-b). However, the proportion of
289 nNOS neurons that were phosphorylated increased significantly at P23 (Fig. 2a-b), simultaneously
290 with the activation of infantile FSH secretion (Fig. 1a), and stayed high until the peripubertal period
291 (P50) (Fig. 2a-b, S1). Intriguingly, P-nNOS immunoreactivity then decreased, reaching infantile
292 values in the OV/MePO of adult male mice (Fig. 2a-b, S1).



293
294
295

Figure 2. The phosphorylation of nNOS coincides with minipuberty in male mice and is independent of infantile gonadal steroids. (a) nNOS (white) and P-nNOS (red) immunoreactivity in forebrain coronal sections

296 at the level of the organum vasculosum of the lamina terminalis (OV) in intact male mice at postnatal days 7 and
 297 12 and in intact and orchidectomized (ORX; carried out on P10) mice at P23. (b) Bar graphs illustrate the
 298 percentage of nNOS-immunoreactive neurons positive for P-nNOS during postnatal development (P7,12, 23,
 299 30, 50, 90) in the OV in intact male mice and at P23 in ORX mice. P-nNOS levels are compared across all
 300 developmental stages (one-way ANOVA with Tukey's post-hoc test). Values after ORX are independently
 301 compared to P23 values (unpaired t-test). Asterisks indicate comparisons to P7 P-nNOS levels unless otherwise
 302 indicated by a bar. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Values indicate means \pm SEM. $N > 3$ independent
 303 litters. (c) Serum LH, (d) testosterone and (e) estradiol levels at P23 in intact and ORX male mice. Values after
 304 ORX are independently compared to P23 values in intact male mice (unpaired t-test for c, e and Mann-Whitney
 305 U test for d). Values indicate means \pm SEM. $N > 3$ independent litters.

306

307 **nNOS is key for the timely activation of the HPG axis at minipuberty**

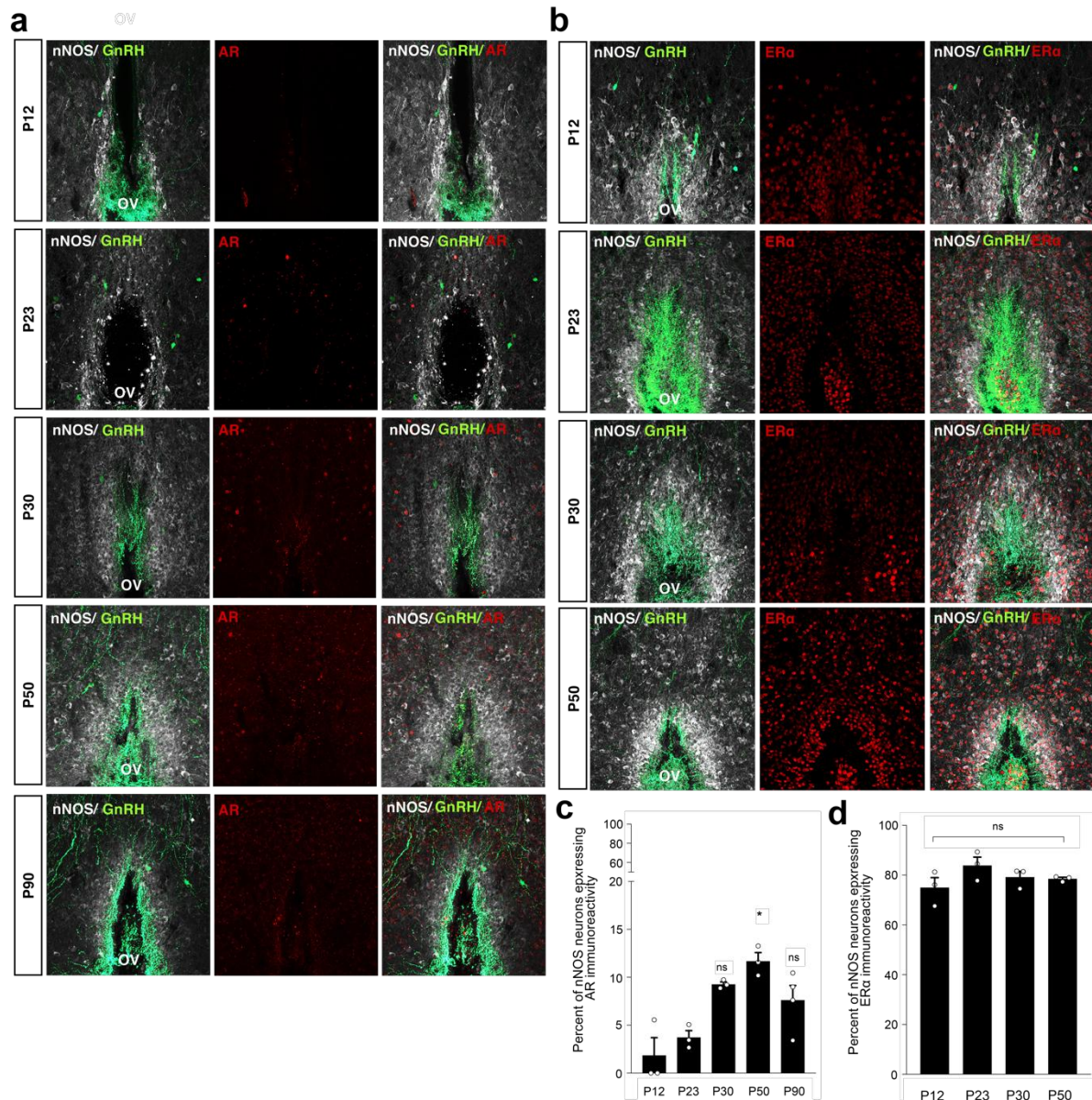
308 In female mice, nNOS deficiency is associated with exacerbated minipuberty, with
 309 significantly higher LH and FSH values at P12 (Chachlaki et al., 2022). We thus examined the same
 310 parameters in male nNOS knockout mice. Gonadotropin measurements in infantile nNOS-deficient
 311 male mice revealed that while the FSH levels were decreased at P23 when compared to wild-type
 312 littermates (Fig. 1a), LH levels showed a precocious peak at P23 (Fig. 1b). Interestingly, the
 313 decreased FSH levels in nNOS knockout mice at P23 were accompanied by an induction of *Fshb*
 314 transcripts in the pituitary (Suppl Fig.2). At the same time, circulating inhibin B increased (Fig. 1c)
 315 while AMH levels remained unchanged (Fig. 1d), supporting an inhibin B-mediated repression of the
 316 release of FSH by gonadotrophs during minipuberty in mutant males (Fig. 1a,c), similar to that seen in
 317 females (Chachlaki et al., 2022). In contrast, pituitary *Lhb* and *Gnrhr* mRNA levels were unchanged
 318 in mutant mice (Suppl Fig.2). The altered FSH release at the peak of minipuberty in mutant mice was
 319 not accompanied by any change in transcriptional profile (Suppl Fig. 3a, b) or testicular histology
 320 (Suppl Fig. 3c), and did not translate into any alteration in circulating testosterone, estradiol or
 321 progesterone levels during infantile development (Fig. 1e-g). However, peripubertal (P50) LH levels
 322 in male mutants were lower than in their wild-type littermates (Fig. 1b), and associated with lower
 323 serum estradiol and progesterone levels, at a time when wild-type levels of the latter show a tendency
 324 to increase (Fig. 1f, g). Examination of circulating testosterone levels revealed a delay in the postnatal
 325 increase of testosterone; nNOS-deficient mice showed lower testosterone levels at P50 than wild-type
 326 mice ($p=0.07$) (Fig. 1e). Together with the delay in other maturational processes such as
 327 balanopreputial separation (Chachlaki et al., 2022), these results suggest a shift in the gonadotropin-
 328 gonadal-steroid profile in the absence of nNOS activity in male mice during postnatal development.

329

330 **Minipubertal nNOS activity in males is influenced by extragonadal estrogens acting through** 331 **ER α**

332 In females, the phosphorylation and activation of nNOS in the OV/MePO appears to be
333 mediated by the action of estrogens following the minipubertal activation of the ovaries (Chachlaki et
334 al., 2022). To verify if gonadal activity at minipuberty also plays a role in preoptic nNOS activation in
335 males, we examined the expression of AR in the OV/MePO of wild-type animals. Surprisingly,
336 immunofluorescence for AR in the OV/MePO was very low or absent during the infantile period and
337 only rose after weaning, during the juvenile period (Fig. 3a, c). Both *Ar* mRNA and protein levels
338 reached a peak during the peripubertal period at P50, but even then, only 10% of nNOS neurons
339 expressed AR (Fig. 3a, c, S4). Equally unexpectedly, when we orchidectomized male wild-type pups
340 at P10 and quantified P-nNOS levels at P23, the already low circulating testosterone flatlined (Fig. 2
341 d) but both P-nNOS expression at P23 (Fig. 2a, b) and circulating LH levels remained unaffected (Fig.
342 2c). Altogether, these data disqualify gonadal testosterone production as the main trigger for nNOS
343 activation during early postnatal development.

344 In stark contrast, >70% of nNOS neurons in the OV/MePO expressed ER α throughout
345 postnatal development, starting as early as P12 (Fig. 3b, d), as in females (Chachlaki et al., 2017b).
346 Orchidectomy at P10 had no effect on circulating estrogens at P23 in male mice, and *Esr1* mRNA
347 expression in the POA did not change during the prepubertal period (Fig. 2e, S4). We therefore tested
348 the possibility that the increase in P-nNOS levels during postnatal development was mediated by ER α
349 by selectively blocking ER α activity between P10 and P23 using the daily intraperitoneal injections of
350 the specific ER α antagonist, MPP dihydrochloride. Intriguingly, although MPP treatment had no
351 effect on circulating estradiol or testosterone levels in infantile mice (Fig. 4c, d), they significantly
352 decreased the percentage of nNOS neurons positive for P-nNOS at P23 (Fig. 4a, b), supporting a
353 previously unsuspected role for extragonadal estrogens in the minipubertal activation of nNOS
354 neurons in males.



355

356 **Figure 3. Expression of the androgen receptor and estrogen receptor alpha in the OV/MePO of male mice**357 **during postnatal development.** (a-b) Immunolabeling at the level of the organum vasculosum of the lamina

358 terminalis (OV) of cells expressing nNOS (white), GnRH (green) and (a) androgen receptor (AR; red) or (b)

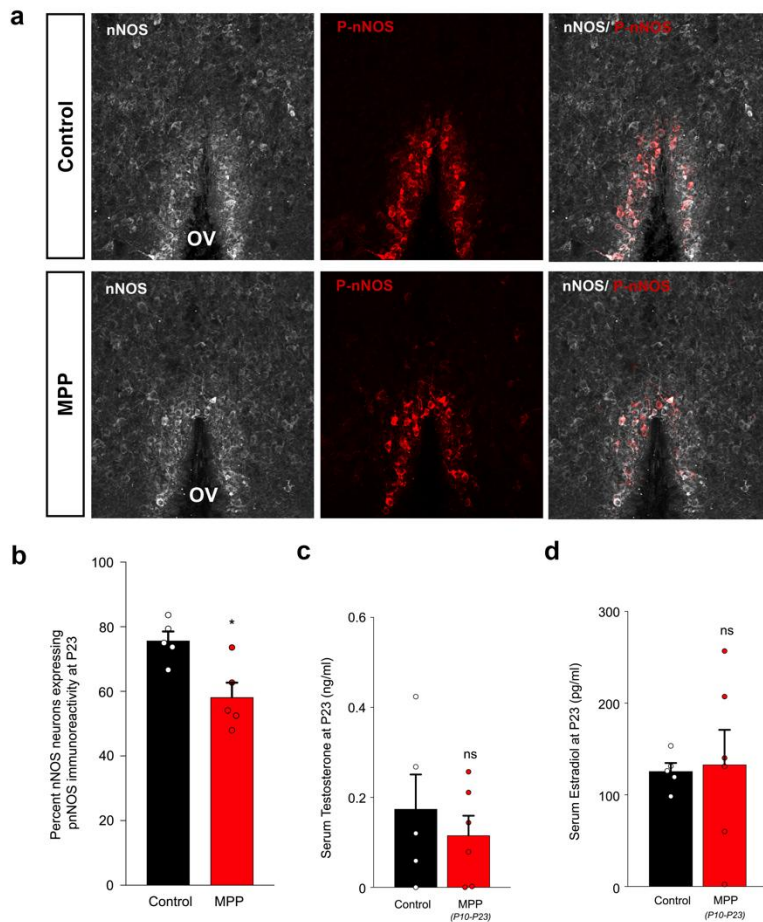
359 estrogen receptor alpha (ERα) in intact male mice at different stages of postnatal development. (c-d) Bar graphs

360 illustrate the percentage of nNOS-immunoreactive neurons also expressing AR or ERα during postnatal

361 development in the OV in intact male mice. nNOS/AR or nNOS/ERα co-expression levels are compared across

362 all developmental stages (Kruskal-Wallis with Dunn's post-hoc test). Values are compared to P12 levels. *

363 P<0.05. Values indicate means ± SEM. N>3 independent litters.



364
 365 **Figure 4. Effect of the ER α antagonist MPP on nNOS phosphorylation and circulating levels of sex**
 366 **hormones in infantile male mice.** (a) Immunolabeling for nNOS (white) and P-nNOS (red) at the level of the
 367 organum vasculosum of the lamina terminalis (OV) of male mice having received MPP dihydrochloride (an
 368 ER α -specific antagonist) or vehicle daily between P10 and P23. (b) Bar graphs illustrate the percentage of
 369 nNOS-immunoreactive neurons positive for P-nNOS at P23 in the OV in control (vehicle-treated) or MPP-
 370 treated (P10-P23) male mice. Values after MPP treatment from P10 to P23 are compared with P23 values in
 371 control male mice (unpaired t-test). * $P < 0.05$. Values indicate means \pm SEM. $N > 3$ independent litters. (c) Serum
 372 testosterone and (d) estradiol levels at P23 in mice having received MPP or vehicle daily from P10 to P23.
 373 Values after MPP treatment are compared to P23 values in control (vehicle-treated) male mice (unpaired t-test).
 374 Values indicate means \pm SEM. $N > 3$ independent litters.

375
 376 Discussion

377
 378 The role of nNOS activity, and subsequent NO release, in the regulation of the
 379 neuroendocrine axis has received increasing attention in recent years (Bellefontaine et al., 2011;
 380 Chachlaki et al., 2017b, 2017a; Delli et al., 2021; Silva et al., 2022; Chachlaki et al., 2022). Recent
 381 findings linking nNOS loss-of-function mutations with congenital hypogonadotropic hypogonadism
 382 (CHH) due to GnRH deficiency in human patients (Chachlaki et al., 2022) have substantiated the

383 infertility observed in nNOS knockout mouse models (Huang et al., 1993; Gyurko et al., 2002;
384 Hanchate et al., 2012; Chachlaki et al., 2022). However, very little is known about how NO controls
385 the first postnatal activation of the GnRH system, i.e. minipuberty, a period considered key for the
386 establishment of adult reproductive physiology.

387 Human studies have already identified important sex differences in the appearance of
388 minipuberty (Kuiri-Hänninen et al., 2011a, 2011b), suggesting that distinct mechanisms could control
389 the postnatal maturation of the HPG axis in the two sexes. In mice, minipuberty has not been
390 extensively studied. In female mice, minipuberty, as defined by a surge in FSH production, begins at
391 around P12 and terminates by the beginning of the juvenile period (around P23) (Prevot et al., 2003;
392 François et al., 2017; Chachlaki et al., 2022). In males, the occurrence of balanopreputial separation,
393 an external sign of sexual maturation, is often confused with the onset of puberty, defined by the
394 ability to reproduce, i.e. the appearance of motile sperm along with high circulating testosterone
395 levels, which only occurs around P55, (Prevot, 2015). Hence, even though minipuberty is shifted in
396 males, reaching a high point at around P23, corresponding to the transition between the infantile and
397 juvenile periods marked by weaning, and terminating at the end of the juvenile stage (around P30), in
398 both male and female mice, minipuberty is an event that is temporally quite distinct from pubertal
399 onset, occurring almost a month earlier. This time window is also in accordance with the gradual
400 increase in GnRH episodic release occurring at the end of the infantile period and reaching zenith
401 values during the juvenile stage, corresponding to male minipuberty, a result that has been also
402 demonstrated in rats (Bourguignon and Franchimont, 1984). As prior reports have also highlighted
403 (Sharpe et al., 1999), FSH concentrations are inversely correlated with inhibin B levels/AMH
404 throughout postnatal development, suggesting that in males, the negative feedback exerted by
405 infantile inhibin B levels controls the timing of minipuberty.

406 Male minipubertal changes are also staggered, with LH levels increasing around the end of
407 the juvenile period (P30), concomitantly with the end of the FSH surge, and dropping back to infantile
408 values during the peripubertal period at P50, in parallel to *Gnrh* mRNA levels. This minipubertal
409 FSH/LH profile may reflect a primary role for FSH, which is involved in the maturation of
410 spermatogonia and Sertoli cell function, in the first stages of male HPG axis development, in contrast
411 to the involvement of LH in later stages of spermatogenesis, when it induces intratesticular
412 testosterone production and supports Leydig cell function (O'Shaughnessy, 2015; Smith and Walker,
413 2015). Indeed, in agreement with early reports (Selmanoff et al., 1977; Jean-Faucher et al., 1978;
414 Pang and Tang, 1984), our results reveal no high infantile testosterone production in males.
415 Interestingly, estradiol levels remained high throughout the developmental stages studied, similar to
416 observations in females (Chachlaki et al., 2022). Although the absolute concentrations we report here
417 match imperfectly with previous reports (François et al., 2017), our finding supports an important role
418 for this so-called “female hormone” in male reproductive development (Cooke et al., 2017) and, in

419 particular, the postnatal maturation of the hypothalamic GnRH neural network, of which nNOS
420 neurons are an integral part.

421 GnRH production during minipuberty (similar to the pubertal GnRH surge) exerts a self-
422 priming effect: the GnRH-induced production of gonadotropins is responsible for gonadal maturation,
423 while steroid hormones resulting from this gonadal maturation exert control on the GnRH secretory
424 pattern. nNOS neurons, which express AR and ER α , seem to be well poised to convey the necessary
425 gonadal information to hormonally-blind GnRH cells. In male mice, as in females (Messina et al.,
426 2016; Chachlaki et al., 2022), the activation of OV/MePO nNOS cells increases along with the rise in
427 circulating FSH levels at P23, indicating that it is coupled with minipuberty. Surprisingly though, the
428 OV/MePO region in the male hypothalamus was devoid of AR immunoreactivity until the early
429 juvenile stage (P23). It gradually increased to reach its highest values peripubertally (P50), but only
430 11.7% of nNOS cells co-expressed AR even at this age. However, the vast majority of nNOS neurons
431 of the OV/MePO expressed ER α as early as P12 both in male and female mice (Chachlaki et al.,
432 2017b), supporting a key role for these neurons in the awakening and priming of the GnRH system at
433 minipuberty. Furthermore, these results suggest that the presence of ER α , but not AR, is important for
434 the integration of hormonal information during the infantile period in both sexes.

435 Estrogenic signals have been shown to be probable determinants for the activity of nNOS
436 cells in female mice during minipubertal and pubertal as well as postpubertal activation of the GnRH
437 system (d'Anglemont de Tassigny et al., 2009; Parkash et al., 2010; Chachlaki et al., 2022), and the
438 ovariectomy of infantile female mice blunts the minipubertal phosphorylation of nNOS protein
439 (Chachlaki et al, 2022). To our great surprise, however, orchidectomy did not have a similar effect on
440 either nNOS phosphorylation in the OV/MePO or the LH profile of infantile male mice. This is in
441 agreement with prior findings in the rhesus monkey showing that the postnatal functional maturation
442 of the male HPG axis is testis- and male-gonadal-steroid-independent (Plant, 1980). Instead, it could
443 be that, at least in males, an extragonadal source provides the estrogenic input necessary to promote
444 the activation of nNOS and subsequently, the GnRH system. In fact, in males, the testes produce only
445 about 20% of circulating estrogens, with the majority of estrogenic content coming from the
446 conversion of testosterone by aromatase (*Cyp19a1*) in the brain or other peripheral tissues such as the
447 adipose tissue, the bones or the skin (Cooke et al., 2017). Interestingly, *Cyp19a1* levels in the POA do
448 not appear to be altered during the prepubertal period or in the absence of nNOS (Suppl. Fig. 4-5), and
449 could continue to contribute to local or circulating estrogen levels. Instead, antagonizing ER α in male
450 mice during infantile development (P10-P23) using MPP dihydrochloride inhibits the minipubertal
451 phosphorylation of the nNOS protein, supporting the idea that estrogens, acting via the ER α -
452 expressing nNOS neurons, are crucial for the maturation of the GnRH system in both female and male
453 mice.

454 Mutant male mice displayed lower FSH levels and a precocious increase in LH levels at the
455 peak of minipuberty. In fact, the increased inhibin B levels in nNOS-deficient mice at P23, a time at

456 which inhibin B (and AMH) were seen to decrease by more than half in wild-type mice, could be
 457 preventing the increase of FSH and thus the normal initiation of minipuberty. At P30, male mutants
 458 showed decreased LH levels while FSH remained unaltered compared to minipubertal values,
 459 whereas in wild-type males, the drop in FSH signifies the end of minipubertal GnRH activation
 460 (Kuiiri-Hänninen et al., 2011b). Considering the importance of pituitary *Pr* downregulation for the
 461 GnRH self-priming effect and the termination of the LH surge in cycling females (Turgeon et al.,
 462 1999; Gordon et al., 2015), the minipubertal increase in *Esr1* and *Pr* pituitary transcript levels we
 463 observed in nNOS-deficient male mice could be associated with the failure of the GnRH system to
 464 terminate minipuberty.

465 Interestingly, the delayed minipuberty in nNOS-deficient male mice was followed by a
 466 peripubertal drop in sex steroid levels (testosterone, estradiol and progesterone), similar to decreased
 467 circulating estradiol levels in female nNOS-deficient mice (Chachlaki et al., 2022), presumably
 468 because deficient GnRH-induced gonadal maturation impacted the later stages of testicular
 469 development, including testosterone production by Leydig cells under the control of LH. This in turn
 470 would attenuate the negative feedback exerted by gonadal steroids on the male GnRH system,
 471 contributing to the improper termination of minipuberty and later acquisition of fertility, as indicated
 472 by delayed balanopreputial separation (an external sign of sexual maturation), hypogonadism and
 473 subfertility in adulthood (Gyurko et al., 2002; Chachlaki et al, 2022). Intriguingly, the immaturity of
 474 the negative feedback operated by gonadal steroids could explain the exaggerated minipuberty
 475 observed in prematurely born girls and boys (Kuiiri-Hänninen et al., 2011a, 2011b).

476 Our previous work identified minipuberty and the subsequent maturation of the HPG axis as
 477 determining factors for not only the acquisition of reproductive capacity, but also for the
 478 establishment of several functions seemingly unrelated to the neuroendocrine axis, such as olfaction,
 479 hearing and even cognition in both sexes (Manfredi-Lozano et al., 2022; Chachlaki et al, 2022) The
 480 current study reveals several important sex differences in the timing of minipubertal activation of both
 481 the nNOS and GnRH systems, as well as an unprecedented common role for estrogens, including
 482 those of extragonadal origin in males, in this process.

483

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485

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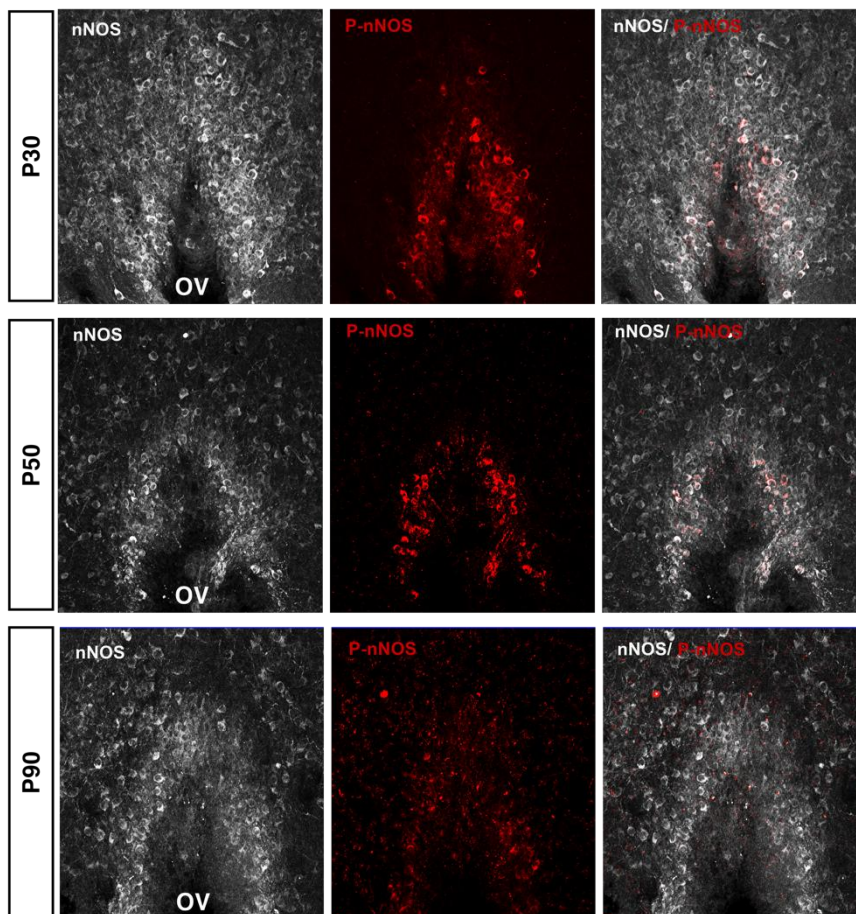
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634 Supplementary Figures

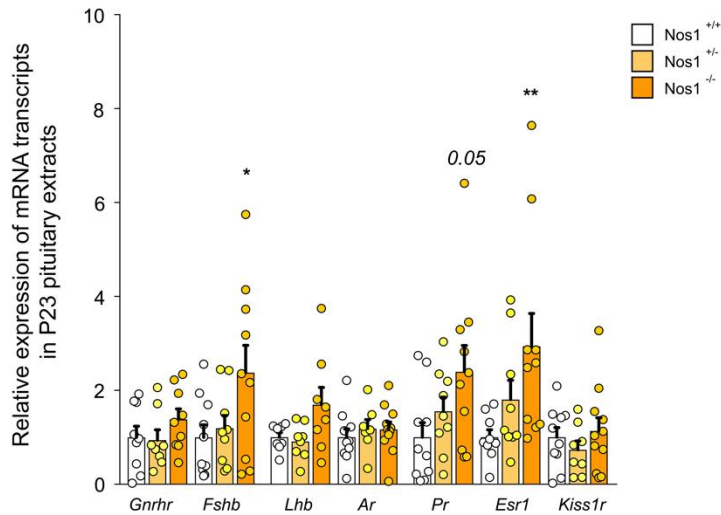
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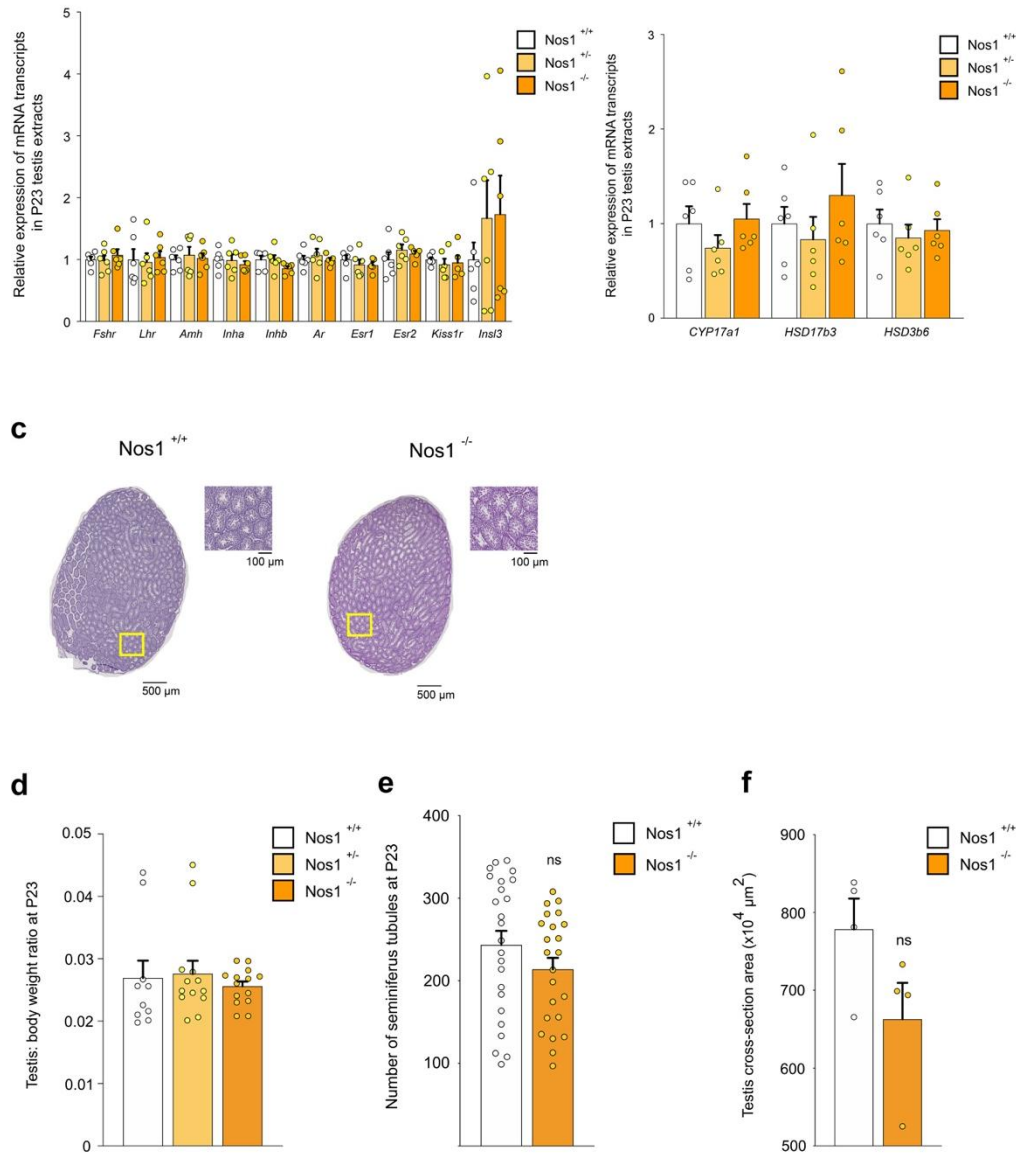
637 **Supplementary Figure 1. Phosphorylation of nNOS in post-minipubertal male mice.** nNOS (white) and P-
 638 nNOS (red) immunoreactivity in forebrain coronal sections at the level of the organum vasculosum of the
 639 lamina terminalis (OV) in intact male mice on postnatal days 30, 50 and 90.



640

641 **Supplementary Figure 2. nNOS deficiency does not alter minipubertal *Gnrhr* expression but affects the**
 642 **transcriptional profile of steroidogenesis-related genes in the male pituitary. *Gnrhr*, *FSHb*, *LHb*, *Ar*, *Pr*,**
 643 ***Esr1* and *Kiss1r* transcript expression in the pituitary at P23 in wild-type and nNOS-deficient male mice. mRNA**
 644 **expression levels were compared to wild-type values (one-way ANOVA with Tukey's post-hoc test for *Gnrhr*,**
 645 ***FSHb*, *LHb*, *Ar* and *Kiss1r* and Kruskal-Wallis with Dunn's post-hoc test for *Pr* and *Esr1*). * P < 0.05; ** P <**
 646 **0.01; Values indicate means ± SEM. N>3 independent litters.**

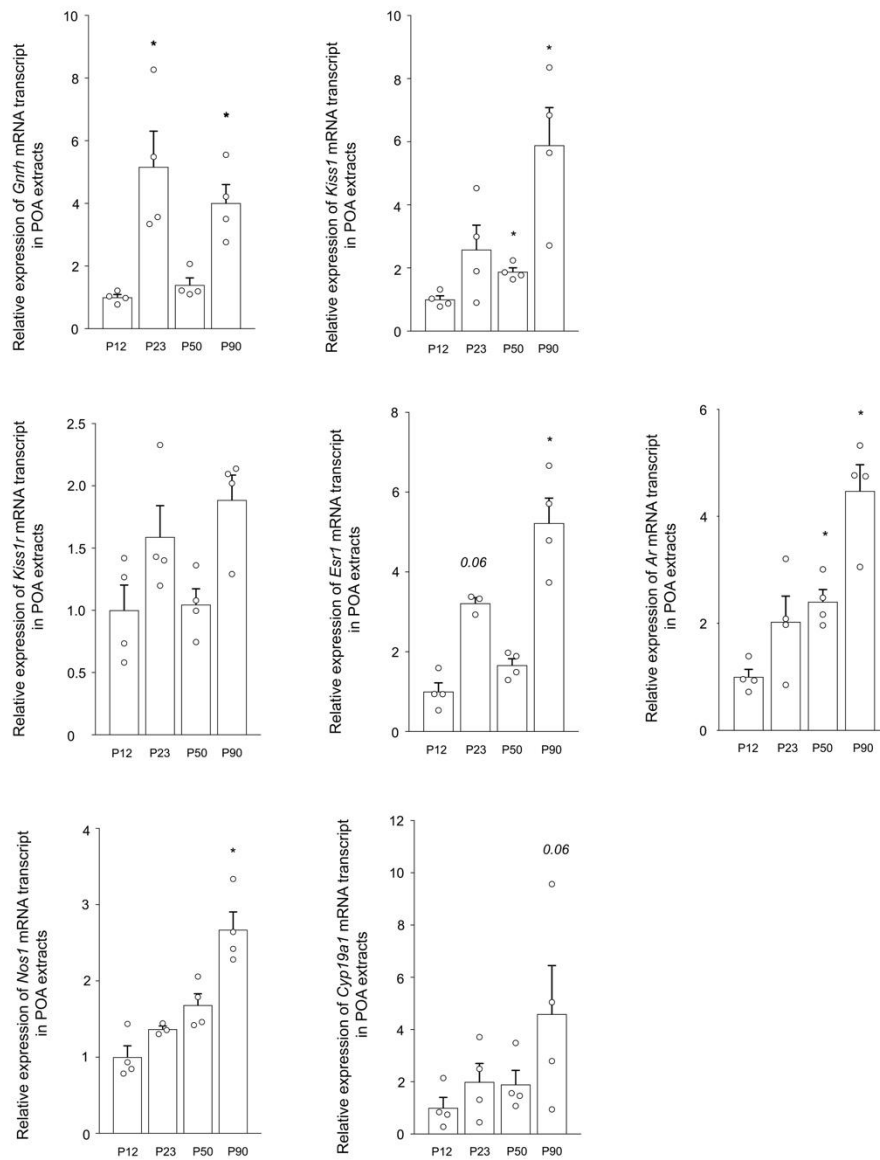
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 649 **Supplementary Figure 3. nNOS deficiency does not alter minipubertal transcriptional profile of**
 650 **steroidogenic genes or basic morphometric parameters in the testis.** (a) *Fshr*, *Lhr*, *Amh*, *Inha*, *Inhb*, *Ar*,
 651 *Esr1*, *Esr2*, *Kiss1r* and *Insl3* and (b) *Cyp17a1*, *Hsd17b3* and *Hsd3b6* transcripts expression in the testis at P23 in
 652 wild-type and nNOS-deficient mice. (c) Representative images of hematoxylin-eosin-stained testis cross
 653 sections from P23 wild-type and nNOS-deficient mice. Scale bar: 500 μ m. Square insets indicate magnification,
 654 scale bar: 100 μ m. (d) Testis-to-body weight ratio, (e) total seminiferous tubule count and (f) Cross sectional
 655 area of the testis in P23 wild-type and nNOS-deficient male mice. Values are compared to wildtype P23 values
 656 (unpaired t-test). Values indicate means \pm SEM. N>3 independent litters.

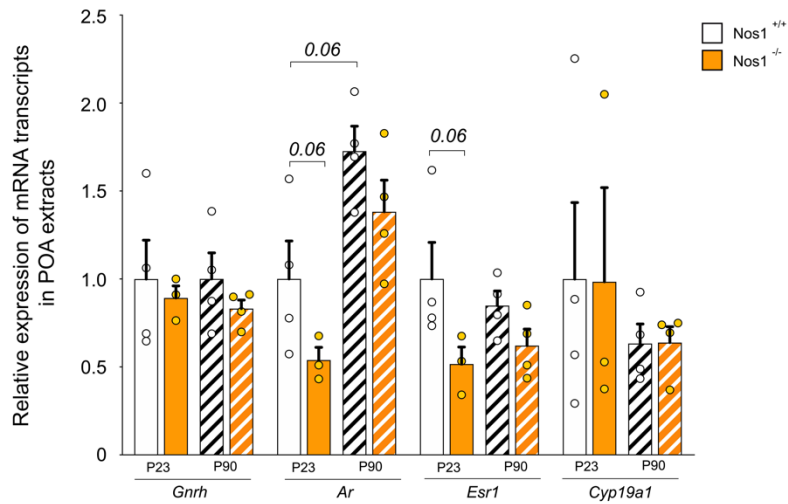
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660 **Supplementary Figure 4 Minipuberty is not accompanied by changes in the mRNA profile of kisspeptin**
 661 **or sex steroid receptors. *Gnrh*, *Kiss1*, *Kiss1r*, *Esr1*, *Ar*, *Nos1* and *Cyp19a1* transcript expression in the preoptic**
 662 **area at P12, P23, P50 and P90 in wild-type mice. mRNA expression levels were compared to P12 values for**
 663 **each timepoint (Mann-Whitney U test). * P < 0.05. Values indicate means ± SEM. N>3 independent litters.**



664

665 **Supplementary Figure 5 nNOS-deficiency does not alter mRNA profile of Gnrh or sex steroid receptors.**666 *Gnrh*, *Ar*, *Esr1* and *Cyp19a1* transcripts expression in the preoptic area at P23 and P90 in wild-type and nNOS-

667 deficient mice. mRNA expression levels were compared to wildtype values for each timepoint (Mann Whitney).

668 Values indicate means \pm SEM. N>3 independent litters.

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