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1 **Genotypic sex shapes maternal care in the African Pygmy mouse, *Mus***
2 ***minutoides***

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9

10 **Abstract**

11 Sexually dimorphic behaviours, such as parental care, have long been thought to be
12 mainly driven by gonadal hormones. In the past two decades, a few studies have
13 challenged this view, highlighting the direct influence of the sex chromosome complement
14 (XX vs XY or ZZ vs ZW). The African pygmy mouse, *Mus minutoides*, is a wild mouse
15 species with naturally occurring XY sex reversal induced by a third, feminizing X*
16 chromosome, leading to three female genotypes: XX, XX* and X*Y. Here, we show that
17 sex reversal in X*Y females shapes a divergent maternal care strategy (maternal
18 aggression, pup retrieval and nesting behaviours) from both XX and XX* females.
19 Although neuroanatomical investigations were inconclusive, we show that the
20 dopaminergic system in the anteroventral periventricular nucleus of the hypothalamus is
21 worth investigating further as it may support differences in pup retrieval behaviour between
22 females. Combining behaviours and neurobiology in a rodent subject to natural selection,
23 we evaluate potential candidates for the neural basis of maternal behaviours and
24 strengthen the underestimated role of the sex chromosomes in shaping sex differences in
25 brain and behaviours. All things considered, we further highlight the emergence of a third
26 sexual phenotype, challenging the binary view of phenotypic sexes.

27

28 **Keywords:** Maternal care, natural sex reversal, third sexual phenotype

29

Introduction

30 Parental care, though not universal across the animal kingdom, is fundamental in many
31 species for offspring' survival and thus, fitness. In mammals, parental care is skewed
32 towards females with obligatory lactation and greater investments, making it one of the
33 most sexually dimorphic behaviours (see [1]). Male care for offspring is rare (5-10% of
34 mammalian species), mainly observed in monogamous species and always associated
35 with biparental care [2,3]. On a proximate scale, this dimorphism as well as other sexually
36 dimorphic traits, was long thought to be a product of gonadal steroid hormones only, acting
37 to shape brain neural circuits [4,5]. In the past two decades, several studies have
38 challenged this view, providing increasing evidence for the direct role of the sex
39 chromosome complement (XX or XY) on sexually dimorphic phenotypes [6–11]. More
40 precisely, genetically modified mice have allowed to dissociate individuals' gonadal and
41 genotypic sexes such as in the four core genotypes (FCG) model, where the *Sry* gene has
42 been deleted from the Y (Y^-) and translocated on an autosome (XX and XY^- females,
43 $XXSry$ and XY^-Sry males) (see [12]). These advances have been crucial to decipher the
44 direct role of both hormones and sex chromosomes on social behaviours [7,10,13–15].
45 However, very few studies have examined how sex chromosomes shape parental care
46 and underlying brain neural circuits [7].

47

48 In rodents for instance, males tend to have more cell bodies and greater fiber densities of
49 vasopressin expressing neurons, a neuropeptide involved in crouching over the pups or
50 maternal aggression among others [16–21]. Interestingly, this dimorphism is likely to result
51 from an imbalance in X chromosome number between the sexes [7,13,22]. Closely related
52 to vasopressin, oxytocin is another substantial neuropeptide for parental care, well-known
53 for its involvement in lactation, but also for enhancing pup retrieval and maternal
54 aggression in females [18,23–26]. However, the impact of the sex chromosome

55 complement on oxytocin in a parental context in the FCG or other mouse models has
56 never been investigated. While the FCG has been a breakthrough in pointing out the value
57 of the sex chromosome complement on parental behaviours, the common view of the
58 system is far from complete considering the complexity of parenting behaviours and the
59 various underlying neural pathways. Additionally, studies have only focused on genetically
60 modified animals so far -which may have some limitations (e.g. absence of *Sry* in XY
61 females), where naturally occurring sex reversal systems subject to natural selection could
62 bring insightful information especially in terms of brain and behaviours' evolution.

63 *In natura*, a handful of rodent species display atypical sex determination systems with loss
64 of the Y chromosome in males, or females carrying a Y chromosome [27]. Among them,
65 the African pygmy mouse, *Mus minutoides*, is particularly interesting: a third sex
66 chromosome, called X*, has evolved from a mutation on the X chromosome, and induces
67 sex reversal of X*Y individuals [28]. Thus, there are three distinct female genotypes in
68 populations, XX, XX* and X*Y, while all males are XY. Sex-reversed X*Y females are fully
69 fertile [29], and have a higher reproductive success than XX and XX* females in laboratory
70 conditions [30]. In addition, when compared to XX and XX* females, X*Y females are more
71 aggressive towards intruders, display reduced anxiety, similar to that of males, and have a
72 greater bite force, also comparable to males [31,32]. These male-typical behaviours
73 observed in X*Y individuals, which are unlikely to be testosterone dependant [33], suggest
74 that the Y chromosome and/or dosage imbalance in X chromosomes masculinize some
75 neural circuits in the brain of *M. minutoides*. The highly contrasted phenotypes between
76 female genotypes thus represent a great opportunity to understand the neural bases of
77 sexually dimorphic behaviours. Furthermore, the dissociation of gonadal and genotypic
78 sexes makes the African pygmy mouse a key species to investigate the effect of sex
79 chromosomes on sex differences in brain and behaviours. In this study, we used an
80 integrative approach to investigate the effect of genotypic sex on maternal care in *M.*

81 *minutoides*, from behaviours to candidate brain neural circuits (Fig. 1). We show, here, that
82 sex reversal in *M. minutoides* influences pup retrieval, maternal aggression, and nest-
83 building behaviours. In rodents, it has been shown that retrieval behaviours and maternal
84 aggression are stimulated by oxytocin expression in the paraventricular nucleus of the
85 hypothalamus (PVN), while nesting behaviours are inhibited by vasopressin expression in
86 the same region [18,24–26,34]. Hence, we then investigated the neuroanatomy of these
87 neural circuits. Based on mouse models, it has also been shown that oxytocin neurons in
88 the PVN are modulated by dopamine neurons in the anteroventral periventricular nucleus
89 of the hypothalamus (AVPV) [26], and that dopamine levels can be positively correlated
90 with *Sry* expression in the brain [35–37]. For instance, *Sry* stimulates the expression of
91 tyrosine hydroxylase (Th), the rate limiting enzyme for dopamine synthesis [35–37].
92 Therefore, we also investigated neurons expressing Th in the AVPV. According to maternal
93 behaviours observed in this study, we expected X*Y females to have a greater number of
94 oxytocin and vasopressin expressing neurons in the PVN as well as a greater number of
95 Th expressing neurons in the AVPV.

96

97 **Material & Methods**

98 Animals, genotype identification and study environment

99 Behavioural experiments were conducted in accordance with European guidelines and
100 with the approval of the French Ethical Committee on animal care and use (No. CEEA-LR-
101 12170) in our laboratory colony (CECEMA facilities of Montpellier University) established
102 from wild-caught animals from Caledon Nature Reserve (South Africa). Approximately 24
103 generations of mice were bred in our colony. Individuals are reared in large (38 L x 26 W x
104 24 H cm) or medium cages (31 L x 21 W x 21 H cm) filled with woodchips bedding, in a
105 room held at 23 °C and with a 15:9 h light-dark cycle. They are supplied with food (mixed
106 bird seeds) and water *ad libitum*. Virgin females are housed in groups of 5-6 in large

107 cages. In our study, either females remained virgins or they were paired at 2-10 months
108 old with a male (aged between 2-19 months old) in a medium cage for breeding and
109 subsequent behavioural assays. Female genotype was identified either by PCR of Sry
110 using total genomic DNA extracted from tail tip biopsies with DNeasy blood and tissue kit
111 (Qiagen) and following Veyrunes et al. [28] protocol, or by karyotyping using bone marrow
112 of yeast-stimulated individuals as previously described [38]. We included X*Y and XX*
113 females from litters of either XX* or X*Y females, and XX females from litters of XX or XX*
114 females. This eliminates potential maternal effects related to the genotype of mothers.

115

116 Behavioural assays

117 Tests were performed on primiparous females (i.e., first litter). X*Y and XX* females were
118 aged 6 ± 2 months old and XX females were aged 5.5 ± 2 months old on average (age
119 range of 3-13 months). Tests were conducted during light cycle, between 10.00 am and
120 6.30 pm, by an experimenter blind to females' genotype. When individuals were assessed
121 in more than one assay, we conducted experiments sequentially by increasing magnitude
122 of stress: we first tested females for pup retrieval, followed by mother-pup interactions and
123 then, nest building, with a 15h gap at least between each test. Pup retrieval and mother-
124 pup interactions assays were filmed with a camera (SONY HDR-CX130E) and
125 subsequently analysed with Observer v5.0.31 and Observer XT softwares (Noldus).
126 Scoring for the nesting and parental care strategy tests were assessed by instantaneous
127 observations. Average litter sizes per female genotype and for each assay are shown in
128 tables S2 and S4.

129

130 *Pup retrieval* experiments were carried out in home cage. Pups were aged 5 ± 2 days old
131 on average. The father and mother were removed while the whole litter was placed at the
132 opposite corner of the shelter with the nest. The assay started once the female was put
133 back under the shelter and recorded until the first pup was retrieved. If no pup had been

134 retrieved within 10 minutes, the assay was ended. A total of 101 females (31 XX, 35 XX* &
135 35 X*Y) were assessed once, on both first approach latency and retrieval probability.

136 *Mother-pup interactions* experiments were carried out in a new cage (38 x 26 x 24 cm)
137 filled with the individuals' own bedding. Pups were aged 4.9 ± 1.7 days old on average. We
138 placed only one pup at the centre of the cage to account for litter size differences between
139 females. We then placed the mother at a corner of the cage and recorded grooming,
140 crouching and carrying behavioural responses during 10 minutes. We assessed 75
141 females (25 per genotype) once, on whether they displace/carry their pup, as well as on
142 duration of grooming, huddling and crouching behaviours.

143 *Nesting* tests were performed in home cage, early evening (~6.00 pm) and nests were
144 scored on the following morning (~10.00 am) for 90 females (28 X*Y, 24 XX and 28 XX*
145 Age of the pups was 6.7 ± 1.9 days old. The father was removed from the cage as well as
146 all environmental enrichments except bedding. We added one nestlet (5x5 cm of
147 compressed cellulose; Serlab, D00009) to provide nesting materials. Individuals received
148 the same nestlet prior to experiment (post-parturition) for habituation. Nest scoring was
149 assessed only once, from 0 to 5 according to Gaskill *et al.* [39] graduation: 0-cellulose
150 unused and no nest, 1- no nest site, 2- flat nest, 3- slightly curved nest, 4- nest with walls,
151 5- fully enclosed nest. We scored the nest accordingly to the graduation above but with
152 more flexibility allowing intermediary scores as well (e.g. 3.5 or 4.5). We also recorded the
153 percentage of nestlet used by females as they do not necessarily use it to build the nest
154 (i.e., they can use their bedding).

155 *Parental care strategy*, or whether mice display biparental care *versus* maternal care only,
156 was investigated for 105 females (29 XX, 37 X*Y and 39 XX*) in home cage. This
157 experiment consisted in observing mates in their home cage, following the birth of the first
158 litter. Hence, no manipulations were made on these individuals. We assessed strategies
159 by characterizing female behaviours towards fathers (i.e., fathers included or excluded

160 from parental care). We made repeated one-off observations (i.e., instantaneous scan) of
161 males' position in the cage and possible physical injuries, from birth to 14 dpp (before pups
162 start to explore outside the shelter) and every 2-3 days. We rated fathers' involvement
163 gradually and increasingly, from 1 to 6: 1= father killed or removed for severe injuries, 2=
164 chased, with aggression stigmata, 3= always in a different lodge, not tolerated in the
165 shelter with the pup(s), 4= tolerated inside the shelter but in the opposite corner, 5= under
166 the same shelter, next to the mother and 6= paternal care observed: huddling, carrying,
167 crouching. Score measurements were not repeated if the father was killed or removed
168 (i.e., score 1 or 2, referred to as high level of maternal aggression).

169

170 Immunohistochemistry

171 We sampled virgin individuals only based on our own ethical considerations as well as for
172 technical limitations : killing mothers would also imply the loss of the litter, while some
173 genotypes, notably XX females, are hard to breed [40]. Eighteen virgin mice (3-5 per
174 genotype including males as a control group) were euthanised with isoflurane 4%
175 anaesthesia followed by decapitation. Brains were collected in 4% PFA and stored for at
176 least 48 hours at 4°C. Brains were sliced at 30 µm using a vibratome (Leica Microsystems)
177 and coronal sections were collected into a PBS-filled container. Floating brain slices were
178 then put in 4x3 well plates (4 wells per individual) and washed three times with Phosphate
179 Buffer Saline (PBS) before staining. We sliced and stained the whole brain to account for
180 any potential divergence from *M. musculus* in the brain anatomy (no data were available
181 on the brain of *M. minutoides*). Brain slices were first incubated for 24h at 4°C or two hours
182 at room temperature (RT) with a Goat coupled Fab anti-mouse IgG blocking buffer
183 (1:1000, Abliance, BI 3013C) diluted in PBS with 0.1% Triton 100X (PBS.T) and ~1% BSA
184 to prevent non-specific binding. Then, slices were incubated for 24-48h at 4°C with mouse
185 anti-Neurophysin I (1:2000, Merck Millipore, MABN844, marker for Oxytocin), rabbit anti-

186 vasopressin (1:1000, Immunostar, 20069) and sheep anti-Th (1:1000, abcam, ab113)
187 diluted in PBS.T and BSA, followed by secondary antibodies : Cy3 coupled Donkey anti-
188 mouse IgG antibody (1:1000, Jackson ImmunoResearch, 715-165-140), Cy5 coupled
189 Donkey anti-rabbit IgG (1:1000, Jackson ImmunoResearch, 711-606-152) and A488
190 coupled Donkey anti-sheep IgG (1:1000, Jackson ImmunoResearch, 713-546-147) diluted
191 in PBS.T and BSA, for 24-48 hours at 4°C. Slices were finally incubated for 3-6 minutes
192 with DAPI (1:10 000, Invitrogen) diluted in PBS and mounted on Polysine® Slides stored at
193 4°C until imaging.

194

195 Imaging and cell counts

196 Brain slices including the PVN and AVPV were identified based on the mouse brain atlas
197 from Franklin and Paxinos [41]. Slices were then processed with a scanner (Zeiss
198 Axioscan) or confocal microscopy (Zeiss, LSM 780) and cell counts (i.e., number of
199 immunoreactive cells) were done blindly and manually on ImageJ, for entire nuclei.
200 Oxytocin and vasopressin neurons' distribution across the whole PVN nucleus were also
201 investigated to account for potential differences in localization between genotypes.

202

203 Statistical analysis

204 All statistical analyses were conducted on R v4.1.2 [42] using *lme4*, *stanarm*, *ordinal* and
205 *multgee* packages (see [43,44] for data and scripts, respectively). Pup retrieval was
206 investigated with Bayesian (generalized) linear mixed models, incorporating the age of
207 pups as a random effect on the intercept. We fit our models to assess retrieval probability
208 and visit latency using a $\log(x+1)$ -transformation to correct for a right-skewed distribution
209 (many latencies at 600s). We ran four Markov chains of 2000 iterations each, removed the
210 first 1000 (warm-up) and kept the second half to estimate our parameters. Both our
211 models checked convergence recommendations ($\hat{R} < 1.1$). Post-hoc comparisons were

212 made using *emmeans* package to contrast each genotype. Point estimates reported per
213 genotype are medians (back transformed from the log (x+1) scale) and median-based
214 contrasts with 89% Highest Posterior Density (HPD) intervals excluding one were
215 considered as truly different (89% HPD is the standard interval for Bayesian models and
216 analogous to 95% confidence intervals). Mother-pup interactions were also analysed with
217 Bayesian (generalized) linear mixed models with pups' age as a random effect and
218 following the same steps as stated above. We log(x+1)-transformed duration responses to
219 correct for a left-skewed distribution (many durations at 0s) and post-hoc comparisons
220 were made to contrast genotypes. Nesting scores were analysed with a cumulative logit
221 model (clm) and probabilities for nest quality per genotype were retrieved accordingly. XX
222 was set as the reference group and we made multiple pairwise comparisons (*emmeans*)
223 with adjusted p-value to assess each genotype effect. We did not include the age of pups'
224 as a random effect due to computational limitations. Parental care strategy was assessed
225 with a population-averaged ordinal model to account for repeated measures and possible
226 correlation between observations using the *ordLORgee()* function from *multgee* package
227 [45]. Probabilities were then retrieved using a back-transformation of parameters
228 estimation according to a cumulative logit link. Cell counts for immunostaining assays were
229 analysed using the non-parametric Kruskal Wallis test for multiple group comparisons.

230

231 **Results**

232 **Higher retrieval efficiency in X*Y females**

233 During pup retrieval experiments, X*Y females approached their pup(s) significantly faster
234 than XX and XX* females (Fig. 2a, Fig. S1, table S1 in [46]), with a median visit latency at
235 51.7s [33.6-73.8 89% HPD] versus 156.5s [94.5-223.3 89% HPD] for XX and 112s [71.5-
236 158.6 89% HPD] for XX* females. Interestingly, 35.5% of XX females did not visit their
237 pup(s) while only 17.1% of XX* females and 11.4% of X*Y did not respond (table S2 in

238 [46]). To confirm that differences were not driven by the absence of response (i.e., visits),
239 we reanalysed latencies only on females visiting their pup(s), and we found the same
240 results (Fig S2 in [46]). Additionally, X*Y females were significantly more likely to retrieve
241 their pup(s) when compared to XX and XX* females, with an estimated retrieval probability
242 of 0.87 [0.77, 0.95 89% HPD], while XX and XX* had a respective probability of 0.47 [0.32,
243 0.63 89% HPD] and 0.55 [0.42, 0.70 89% HPD] only (Fig. 2b, tableS1 in [46]). Hence, X*Y
244 females are more efficient at pup retrieval than XX and XX* females.

245

246 **Poor nest quality in X*Y females**

247 All females showed nesting behaviours as no nest score below 3 was recorded. However,
248 X*Y females were more likely to build poor quality nests in contrast to both XX (Fig. 2c, $z =$
249 -3.5 , $p = 0.001$) and XX* females ($z = -4.61$, $p < 0.001$). They had a probability of 0.37
250 (highest probability) for building a slightly curved nest (i.e., score 3) while they had a 0.15
251 probability, for making a fully enclosed nest (i.e., score 5). Conversely, XX and especially
252 XX* individuals were more likely to build high quality nests, with a respective probability of
253 0.51 and 0.70 for a fully enclosed nest. This also indicates a relatively high consistency in
254 nest-building behaviour for XX* females. Though XX* had a higher probability than XX
255 individuals for building high quality nests, they show similar behavioural trends (Fig. 2c, $z =$
256 -0.78 , $p = 0.35$). Interestingly, most X*Y females (89.3%) did not use the nestlet to build a
257 nest but used their own bedding, in contrast to 50% for XX and 42.9% for XX*. We
258 therefore tested for a relationship between nest quality and nestlet usage (i.e., disinterest
259 for the nestlet or poor learning) in X*Y females and did not find any correlation ($\chi^2 = 12.04$,
260 $p = 0.12$). X*Y females are thus less efficient at nest building when compared to XX and
261 XX* females.

262

263 **Genotypic sex does not impact mother-pup interactions**

264 Most females interacted with their pup and there were no significant differences in
265 interaction durations nor probabilities (table S3, Fig. S3-4 in [46]). X*Y, XX and XX*
266 females spent in median 7.9s [3.07-13.2 89% HPD], 11.2s [4.65-18.8 89% HPD] and 15.9s
267 [7.03-25.8 89% HPD] crouching over the pup, 7.17s [3.48-11.36 89% HPD], 4.96s [2.26-
268 7.97 89% HPD] and 7.30s [3.58-11.64 89% HPD] huddling with the pup, 3.18s [1.36-4.99
269 89% HPD], 4.87s [3.49-7.46 89% HPD] and 5.79s [2.89-8.70 89% HPD] grooming the pup,
270 respectively. In addition, they had respective probabilities of 0.68 [0.42-0.9 89% HPD],
271 0.76 [0.5-0.93 89% HPD] and 0.67 [0.4-0.87 89% HPD] to displace their pup.

272

273 **Genotypic sex influences female parental strategy**

274 X*Y females were on average more likely to show maternal aggression than XX and XX*
275 females, which inversely, were more likely to be involved in biparental care (Fig. 2d, X*Y vs
276 XX, $z=-2.81$, $p=0.005$; X*Y vs XX*, $z=-2.87$, $p=0.004$). Indeed, X*Y females had a highest
277 probability at 0.33 to severely aggress or kill mates (i.e., score 1) in contrast to a 0.19
278 probability to display biparental care (i.e., score 6). Inversely, XX and XX* females had
279 highest probabilities at 0.46 and 0.43 respectively for biparental care in comparison to 0.12
280 and 0.13 probabilities for maternal aggression. Strikingly, XX and XX* females' curves
281 almost overlay with quite similar probabilities in each category, highlighting identical
282 parental care strategy in XX and XX* females.

283

284 **Hypothalamic neural circuits of maternal care**

285 We did not find evidence for difference in the global neuroanatomy of *M. minutoides* PVN
286 in comparison to *M. musculus* [23,41,47]. Vasopressin is expressed in both parvo- and
287 magnocellular neurons, mostly in intermediate regions of the PVN where it is expressed
288 dorsolaterally (Fig. S5, S7-S10 in [46]) and oxytocin is mostly expressed in magnocellular
289 neurons and more homogeneously across the nucleus than vasopressin (midline and
290 rostro-caudal axis; Fig. S6-S10 in [46]). We did not have enough power to statistically

291 compare oxytocin and vasopressin neuron distributions across genotypes but there were
292 no overall striking differences (Fig. S5-S6 in [46]), with high variabilities in count data due
293 to inter-individual variability and small sample sizes. In addition, there were no differences
294 in oxytocin neurons number between genotypes (Fig. 3a,c-S6, $\chi^2 = 0.89$, $p = 0.83$). We did
295 not find differences in the number of vasopressin expressing neurons either (Fig. 3b,c,
296 $\chi^2=0.82$, $p = 0.84$) but there is a tendency for a lower number in X*Y females driven mostly
297 by the lower number of vasopressin neurons in anterior regions (Fig. S5 in [46]). Finally,
298 there were no differences in the neuroanatomy of the AVPV in comparison to *M. musculus*
299 [41,48,49]. Since there is evidence for a sexual dimorphism in the nucleus size in *M.*
300 *musculus* [49,50], we weighted the number of Th expressing neurons by the area of the
301 nucleus and did not find differences in the density of Th neurons between genotypes
302 ($\chi^2=2$, $p = 0.57$), though a tendency for a greater density in X*Y females (Fig. 4).

303

304 **Discussion**

305 The African pygmy mouse is a species with a naturally occurring sex reversal system,
306 involving a dissociation between phenotypic and genotypic sexes in X*Y females. This
307 fascinating system represents a great opportunity to shed light on the impact of the sex
308 chromosomes on sexually dimorphic behaviours such as parental care. By focusing on
309 maternal phenotypes specifically, we show that sex reversal in *M. minutoides* influences
310 pup retrieval, maternal aggression, nest building behaviours and may also impact the
311 maternal dopaminergic circuit in the AVPV nucleus. Our study confirms and further
312 extends evidence on the influence of the sex chromosome complement on parental
313 behaviours and brings promising prospects for its impact on the neurobiology of parenting.

314

315 X*Y females are more efficient at pup retrieval than both XX and XX* females. They first
316 approached their pup faster and showed more consistent retrieval, while XX and XX*

317 showed increased latencies to first approach and retrieve their pup about one out of two
318 times only. Moreover, in a reproductive context, X*Y mothers were highly aggressive
319 towards mates, whereas XX and XX* females were more likely to share parental care.
320 Previous results showed that non-reproductive X*Y females are more aggressive than
321 other female genotypes towards intruder males [31]. Here, we show this enhanced
322 aggressiveness further extends towards the pup's own father (as male partners were killed
323 or needed to be removed for 43.2% of X*Y females (i.e., 16 out of 37), suggesting that X*Y
324 females are more likely to be solitary mothers. Furthermore, X*Y females built poor quality
325 nests (slightly curved), while XX and especially XX* females performed better with fully
326 enclosed nests. It was surprising that almost all X*Y females did not use the nestlet but
327 their own bedding, and the lack of correlation between nestlet usage and nest quality
328 strongly suggests a disinterest for the nestlet in X*Y females. Because nest building is an
329 essential component of maternal care especially in altricial species, to protect the young
330 from potential threats and for thermoregulation, this would thus suggest maladaptation in
331 X*Y females. However, here, experiments were conducted in breeding conditions where
332 temperature is held constant and where the only potential threat is the father (i.e.,
333 infanticide). In addition, we show that X*Y females can assure pups protection through
334 agonistic behaviours (i.e. maternal aggression). Therefore, nest quality might be less
335 essential for these females in breeding conditions, and in the future, it would be worthwhile
336 to compare these behaviours with wild animals in natural populations. All things
337 considered, the divergent behavioural patterns between X*Y and XX / XX* females reflect
338 two distinct maternal strategies shaped by the sex chromosome complement in *M.*
339 *minutoides*. Although there is an unequivocal effect of the sex chromosome complement
340 on some maternal behaviours, there were no differences in mother-pup interactions
341 suggesting that it does not modulate all components of maternal care.

342

343 It is very striking that X*Y females displayed -quite consistently-, divergent maternal
344 behaviours from XX and XX* females: they showed a greater retrieval efficiency, high level
345 of maternal aggression, but also poor nest-building skills as found in males suggesting
346 masculinization (data not shown; [51]). Interestingly, this pattern of co-occurrence of both
347 feminized and masculinized traits in X*Y females was already found in previous studies:
348 while they have functional ovaries and show, moreover, a greater reproductive success
349 than both XX and XX* females (e.g. higher ovulation rate, bigger litter sizes) [29,30], they
350 also display male-like reduced anxiety behaviours, they are more aggressive, and have a
351 greater bite strength than XX and XX* females [31,32]. All things considered, the constant
352 divergence between X*Y on one side and XX and XX* females on the other side highlights
353 that the association of a feminizing X* and a male-specific Y chromosome has given birth
354 to a third sexual phenotype in the African pygmy mouse.

355

356 The differences of maternal care behaviours between female genotypes raise questions
357 concerning the genetic basis of maternal strategies in *M. minutoides*. Several hypotheses
358 are discussed here. First, the divergent behaviours of the X*Y females suggest an
359 influence of the Y chromosome and one prime candidate among this gene-poor
360 chromosome is *Sry*, which is expressed in the brain of X*Y females [29]. In male rats and
361 mice for instance, *Sry* expression in the brain regulates some neural pathway such as
362 dopaminergic neurons (e.g. positive regulation of tyrosine hydroxylase, Th, expression in
363 the *substantia nigra*), which is likely to influence behaviours in a male-specific manner [35-
364 37]. Secondly, there could also be an influence of the female specific X* chromosome.
365 However, since XX* females behave like XX females, this would imply maternal X*-biased
366 chromosome inactivation (X*CI), or parental imprinting of X-linked genes [52,53], with
367 preferential expression of the paternal X alleles. There is no evidence for skewed X*CI in
368 *M. minutoides* based on cytological investigations of embryos fibroblast cell cultures [54],

369 but next generation sequencing technologies with a special focus on allele-specific
370 expression in the brain would be necessary to confirm or refute this finding, and therefore
371 help clarify hypotheses. Thirdly, another hypothesis to correlate with divergent maternal
372 strategies is X dosage (i.e., copy number of X and X* chromosomes; 2 copies in XX and
373 XX*, and only one copy in X*Y females). In the house mouse, hundreds of autosomal
374 genes are under the influence of X dosage [55], such as vasopressin involved in nesting
375 behaviours [7,13,19,34]. In addition, a few X-linked genes (3-8%), such as *Kdm6a* involved
376 in gene expression regulation, escape inactivation in mice and have also been suggested
377 to influence sexual phenotypes ([12,15,56]). Finally, one might also suppose an epistasis
378 interaction between the X* and the Y chromosome. In *Drosophila melanogaster* for
379 instance, it has been shown that a few X-linked genes involved in pheromone detection
380 are regulated by the Y chromosome, which is likely to impact reproductive phenotypes
381 [57].

382 Interestingly, some of our behavioural observations are in contradiction with previous
383 findings on the FCG, which may give insight into their genetic bases. Contrary to X*Y
384 females in *M. minutoides*, sex-reversed XY- females in the FCG displayed similar nesting
385 behaviours to XX females, but had impaired retrieval capabilities: they retrieved less pups
386 and belatedly when compared to XX females [7]. Considering nest building, in the FCG
387 model, the authors suggested that the poor nesting skill in XY-*Sry* and XX*sry* males
388 compared to XX and XY- females were due to the expression of *Sry* and/or the secretion
389 of perinatal androgens, which masculinize the underlying neural circuits [7]. In our case,
390 X*Y females built poor quality nests as well, while they do not have differences in
391 gonadal/adrenal hormonal levels compared to the other female genotypes (testosterone
392 and estradiol; [33]) but they do express *Sry* in their brain [29]. Therefore, *Sry* is a good
393 candidate to correlate with impaired nesting behaviours in these females. Considering pup
394 retrieval, results in the FCG suggested that the Y chromosome and/or X dosage imbalance

395 was necessary to alter retrieval phenotypes. Here, we show that X*Y females perform
396 better at pup retrieval even though they have one copy of the X* chromosome, a Y
397 chromosome, and express *Sry* in their brain [29]. Therefore, either the impact of Y-linked
398 genes (including *Sry*) is different between FCG mice and the African pygmy mouse, either
399 it is an X* chromosome effect, or an epistasis interaction between the X* and the Y
400 chromosome. Although the genetic bases remain unknown, our results show that sex
401 reversal in the African pygmy mouse does not seem to alter maternal care quality, contrary
402 to expectations based on the FCG. This discrepancy further illustrates the need to
403 consider not only genetically modified laboratory mice but also non-model species with
404 alternative phenotypes shaped by natural selection -such as observed in *M. minutoides*-,
405 to better understand the genetic and neural bases of behaviours.

406

407 In mammals, oxytocin and vasopressin are crucial neuropeptides that intervene in the
408 initiation and maintenance of parental care [58]. In rodents, oxytocin expression in the
409 PVN induces pup retrieval and maternal aggression [18,24–26,59] while vasopressin,
410 which can also mediate maternal aggression, has been shown to inhibit nest building
411 [18,34]. Moreover, vasopressin has been shown to be influenced by X chromosome
412 number, with greater expression in individuals with a single X chromosome [7,13,19].
413 Hence, oxytocin and vasopressin were prime candidates to explain the divergent maternal
414 behaviours in *M. minutoides*, and a greater number of vasopressin and oxytocin neurons
415 in the PVN of X*Y females in comparison to XX and XX* females could have been
416 expected (Fig. 1). However, we did not find such differences between females nor with
417 males, emphasized by the high variability in count data (i.e. indicative of low power; Fig.
418 3a,b). The lack of differences in oxytocinergic and vasopressinergic systems global
419 anatomy in the PVN suggests no differences in oxytocin and vasopressin levels.
420 Nonetheless, larger sample sizes and the functionality of the neuroendocrine networks as

421 well as the hormonal output should be tested to confirm or refute this hypothesis (e.g.
422 quantification of gene and protein expression in the PVN, blood hormonal levels measured
423 over time) [19,26,34]. In addition, we tested virgin females while parental neural circuits
424 are known to evolve with sexual experience [25,26,58,60]. Differences in oxytocin and/or
425 vasopressin between females may thus arise at first pregnancy.

426 Another interesting brain region to look at was the AVPV. It has been shown that neurons
427 expressing tyrosine hydroxylase (Th) in the AVPV stimulate oxytocin expressing neurons
428 in the PVN and consequently pup retrieval in females [26]. Moreover, these neurons are
429 sexually dimorphic with a greater number in females than males already in virgin
430 individuals [26,49]. Our results do not support the sexual dimorphism described in *M.*
431 *musculus*, however we show a tendency for a greater density of Th neurons in X*Y
432 females, which is also supported by preliminary results on *Th* mRNA expression levels
433 (RNAseq and RT-qPCR) in the whole brain of *M. minutoides* (unpublished data).
434 Therefore, the tendency for Th may also be consistent with pup retrieval results and it
435 would be worth exploring further this potential link between sex chromosomes and the
436 regulation of parenting through the dopaminergic system.

437 Finally, it is noteworthy that parenting is a complex behaviour that is regulated by diverse
438 neural pathways and neuromodulators. In mice for instance, nesting behaviours have also
439 been shown to be inhibited by another peptide, the neuropeptide Y, whose expression is
440 sexually dimorphic [61,62]. It is thus likely that the sex chromosome complements in *M.*
441 *minutoides* act on various neural circuits to shape maternal behaviours, and future studies
442 should integrate other brain regions and neuromodulators, to help understand the neural
443 basis of maternal strategies. Because we work on a wild non-model species, we have
444 ethical considerations, which restrain the establishment of a causal link between brain
445 nucleus, peptides and behaviours: we have small sample sizes, use virgin individuals only,
446 and face technical limitations (e.g. stereotaxic surgery has never been performed on this

447 species). Nonetheless, inceptive investigations of the neuroanatomy provide an essential
448 basis to describe and understand the evolution of the brain and behaviours, as illustrated
449 here in a species with highly contrasted phenotypes.

450

451 Combining behaviours and the neuroanatomy of parenting, we highlight the emergence of
452 a third sexual phenotype in a wild species. While the relative impact of each sex
453 chromosome on maternal behaviours is yet to be determined, our results bring exciting
454 advances to research on both the neural basis and the impact of the sex chromosomes on
455 natural behaviours. Furthermore, research on multiple sexual phenotypes in other wild
456 species with unusual sex determination system as described here, in the African pygmy
457 mouse, could help strengthen the challenge of rethinking the stereotypical dichotomous
458 view of sexes.

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473 **Figures**

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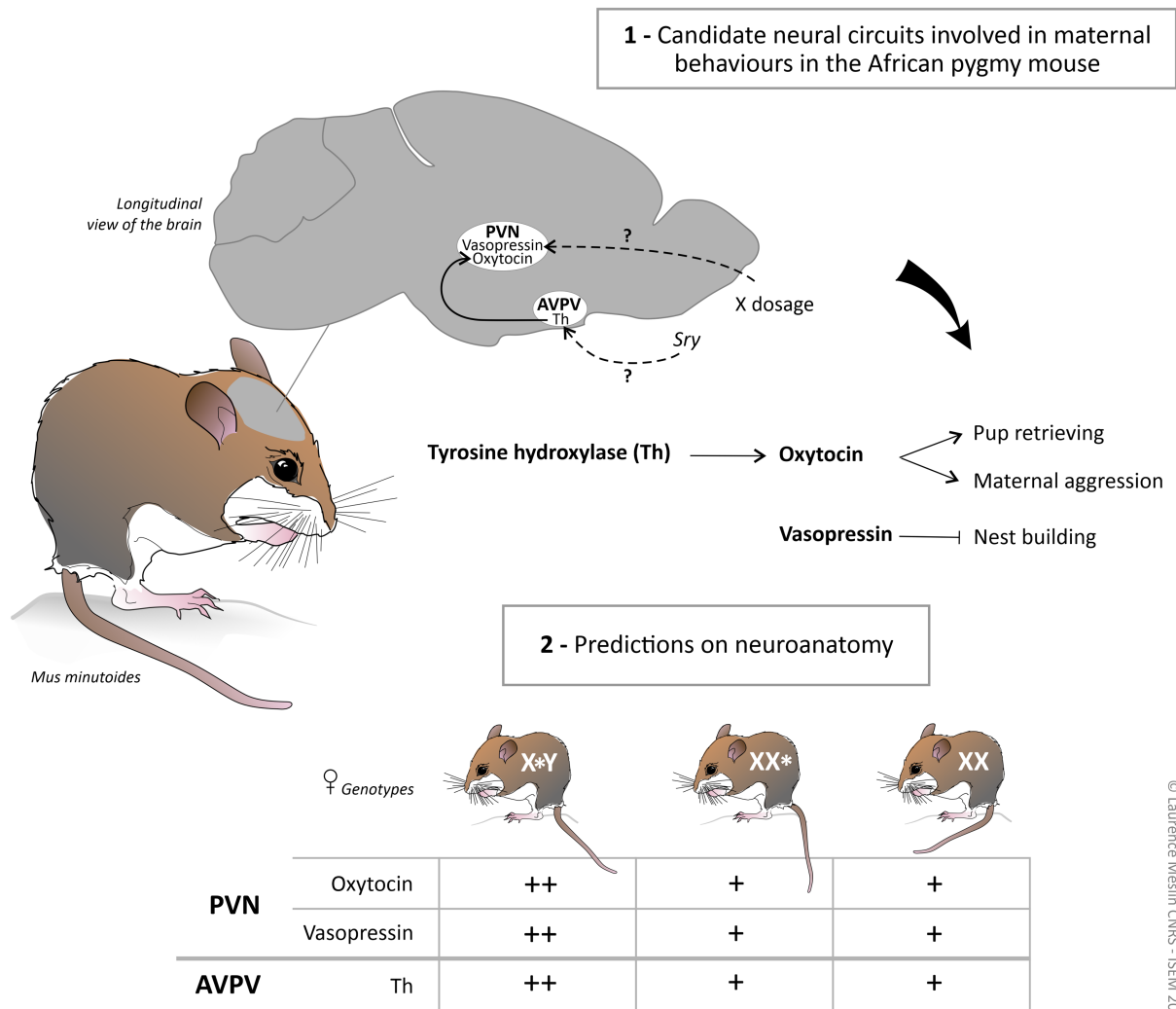
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492 **Figure 1.** Neural investigations and predictions based on maternal behaviours observed in
 493 the African pygmy mouse, and the known influences of some neuropeptides and
 494 neurotransmitters on these behaviours in other rodents. Maternal behaviour experiments
 495 showed that sex-reversal in *M. minutoides* influenced pup retrieval, maternal aggression
 496 and nest building, with X*Y females being different from both XX and XX* females for each
 497 behaviour. We investigated potential candidates for the neural basis of these behaviours
 498 based on *M. musculus* and *Peromyscus* studies: the paraventricular nucleus (PVN) of the
 499 hypothalamus where the expression of oxytocin triggers pup retrieval and maternal
 500 aggression [18,24–26] while vasopressin expression inhibits nesting behaviours [34]. We
 501 also investigated Th neurons (i.e., dopaminergic system) in the anteroventral
 502 periventricular nucleus (AVPV) of the hypothalamus, which have been shown to enhance
 503 retrieval behaviours through the regulation of oxytocin neurons in the PVN (solid arrow)
 504 [26]. Moreover, it has been shown that Th neurons can be regulated by the sex
 505 determining gene, *Sry* (e.g. [36]) and that vasopressin can be influenced by X dosage (e.g.
 506 [7]) (dashed arrows). All things considered, we predicted greater oxytocin, vasopressin and
 507 Th cell numbers / density in the PVN and AVPV of X*Y females, respectively. Predictions
 508 are represented by + symbols. © Laurence Meslin CNRS - ISEM 2022

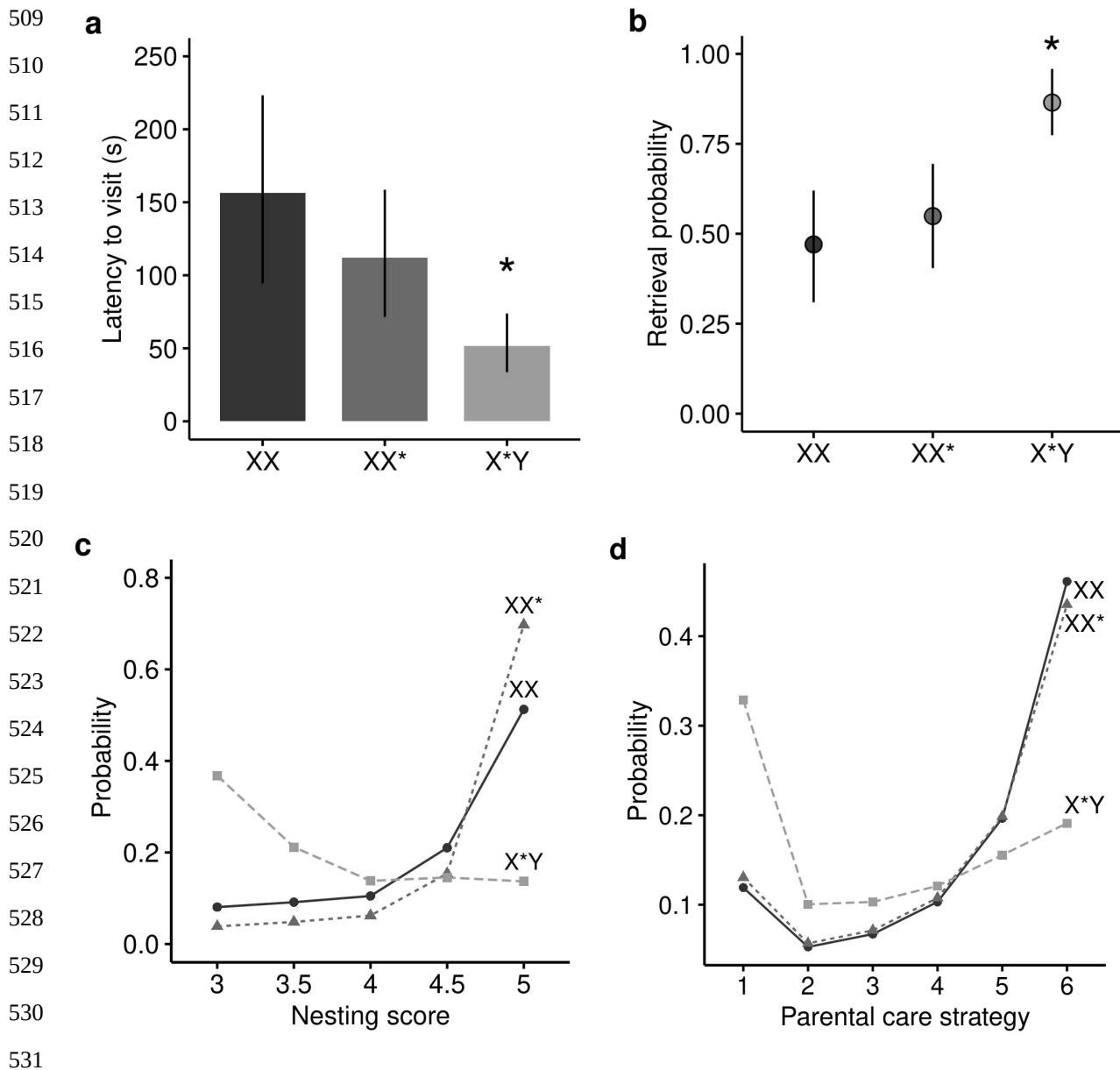
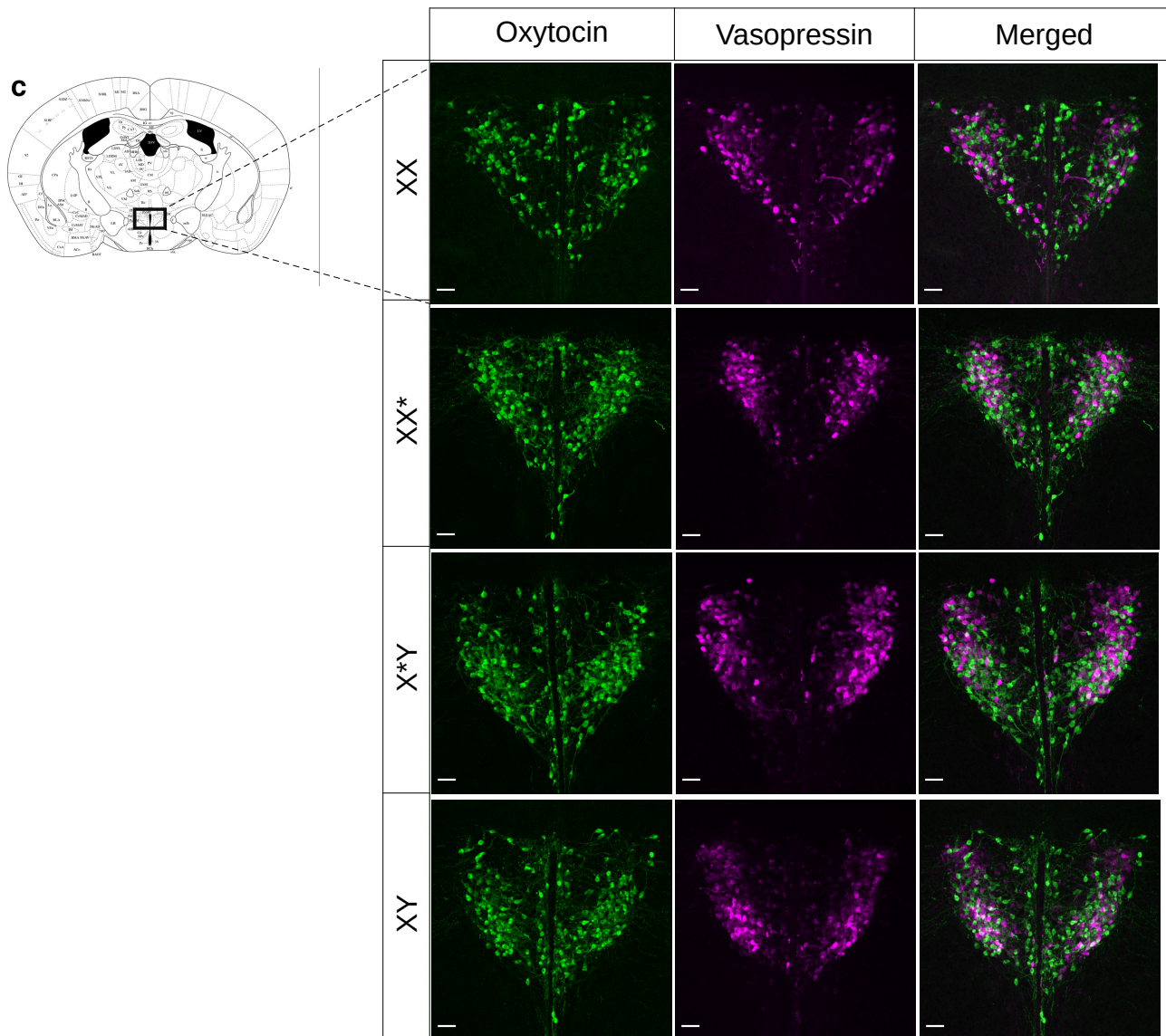
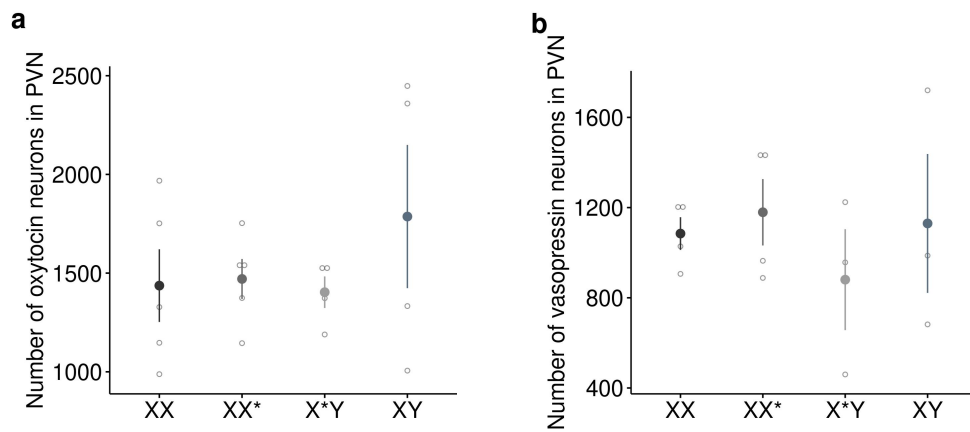


Figure 2. Sex chromosome complements shape maternal behaviours in the African pygmy mouse. **a,b**, Pup retrieval. Estimates of first visit latency and retrieval probability per genotype. Values are medians with 89% HPD intervals. Latency to first visit and probabilities are retrieved from a back-transformation of our parameters. *Asterisks indicate significance of the genotype effect : X*Y females show a significantly reduced visit latency and a higher retrieval probability in contrast to XX and XX* females (median-based contrasts with 89% HPD intervals are shown in table S2). $n_{XX}=31$, $n_{XX^*}=35$, $n_{X^*Y}=35$ **c**, Likelihood curves of nest quality depending on females' genotype. Nest quality estimates range from 3 (slightly curved nest) to 5 (fully enclosed nest) as no values below 3 were recorded. X*Y females build poorer quality nest than both XX ($p=0.001$) and XX* females ($p<.0001$). $n_{XX}=24$, $n_{XX^*}=28$, $n_{X^*Y}=28$ **d**, Likelihood curves of parental care strategies regarding fathers involvement inside the nest from 1 : no involvement of fathers and high level of maternal aggression to 6 : biparental care. $n_{XX}=29$, $n_{XX^*}=39$, $n_{X^*Y}=37$. X*Y females are more likely to display maternal aggression than both XX ($p=0.005$) and XX* females ($p=0.004$), which likely share care with males.

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Figure 3. No genotypic sex effect on oxytocin and vasopressin neurons' number in the PVN of virgin individuals. **a,b**, Total number of oxytocin and vasopressin expressing neurons in the PVN of virgin XX, XX*, X*Y females and XY males. Oxytocin : $n_{XX}=5$, $n_{XX^*}=5$, $n_{X^*Y}=4$, $n_{XY}=4$. Vasopressin : $n_{XX}=4$, $n_{XX^*}=4$, $n_{X^*Y}=3$, $n_{XY}=3$. Data are means \pm s.e.m and raw counts are also represented by empty dots. **c**, Oxytocin and vasopressin staining in the PVN of virgin African pygmy mice and associated *M.musculus* brain slice reference (black rectangle represents the PVN area, modified from Franklin & Paxinos [41]). Scale bars 50 μ m.

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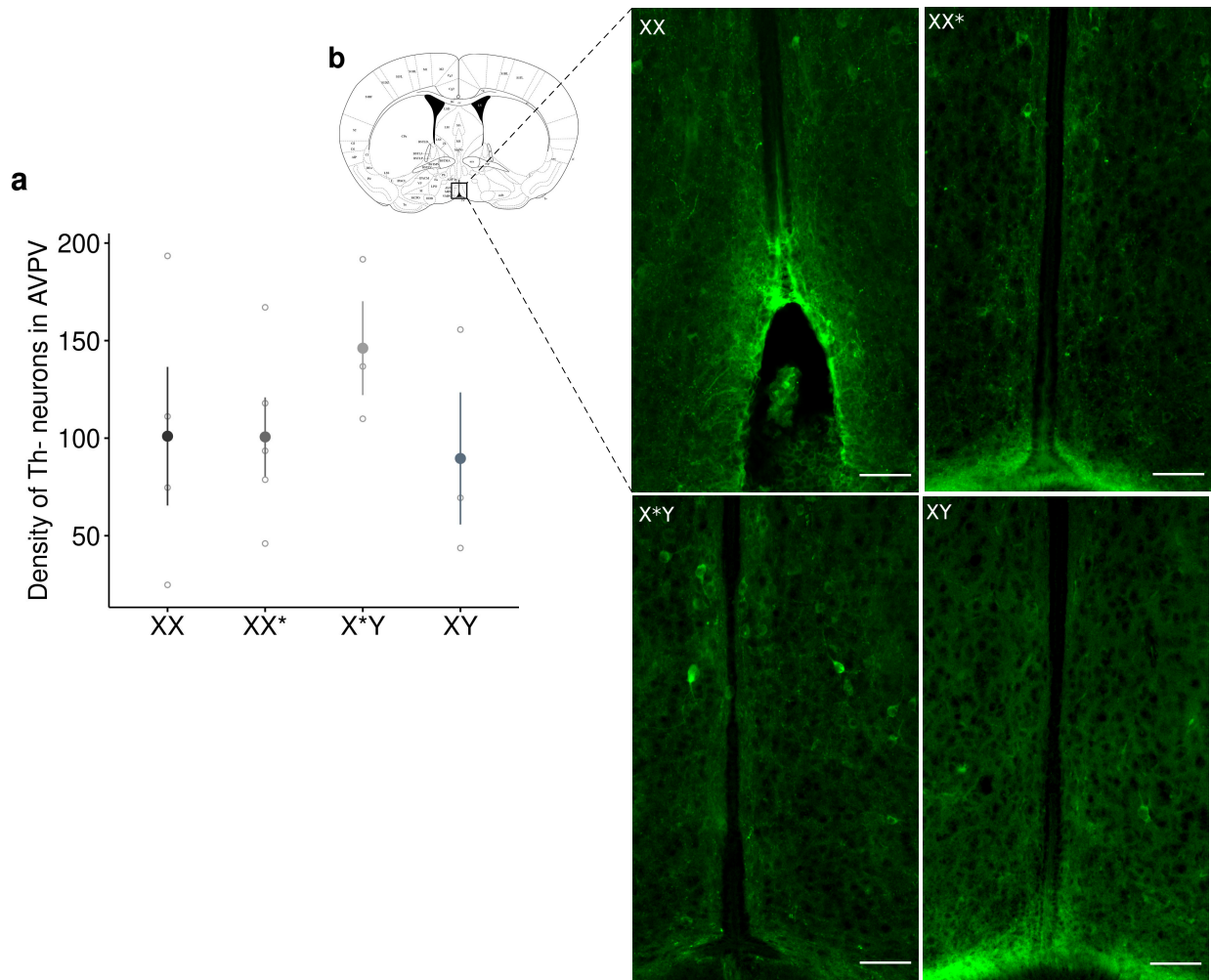


Figure 4. Tendency for a greater Th neuron density in the AVPV of X*Y females. **a**, Density of Th- expressing neurons in the AVPV of virgin XX, XX*, X*Y females and XY males: number of total Th neurons weighted by the sum of the area (mm²). $n_{XX}=4$, $n_{XX^*}=5$, $n_{X^*Y}=3$, $n_{XY}=3$. Data are means \pm s.e.m and raw counts are also represented by empty dots. **b**, Th staining in the AVPV of virgin African pygmy mice and associated *M.musculus* brain slice reference (black rectangle represents the AVPV area, modified from Franklin & Paxinos [41]). Scale bars 50 μ m.