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

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abstract

Parental care, one of the most sexually dimorphic behaviour in mammals, was long thought to be driven mostly, if not exclusively, by gonadal hormones. Over the past two decades, very few studies have challenged this view, highlighting the direct influence of the sex chromosome complement (XX vs XY). The African pygmy mouse, *Mus minutoides*, is a wild mouse species with a naturally occurring sex reversal due to a third, feminizing X* chromosome, leading to three female genotypes: XX, XX* and X*Y. Here, we show that the sex reversal in X*Y females shapes a divergent maternal care strategy from both XX and XX* females, rather than altering care quality. In addition, the sex reversal is likely to impact the dopaminergic system in the anteroventral periventricular nucleus of the hypothalamus, consistent with one component of maternal care: pup retrieval. Combining neurobiology and behavioural ecology in a wild mouse species subject to natural selection, we evaluate here the neural basis of maternal behaviours and strengthen the underestimated role of the sex chromosome in shaping sex differences in brain and behaviours. Furthermore, we highlight the emergence of a third sexual phenotype, challenging the binary view of sexes.

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

Introduction

Parental care, though not universal across the animal kingdom, is fundamental in many species for offspring' survival and thus, reproductive success. Among mammals, parental care is skewed towards females which invest more notably as a primary result of an obligatory lactation. Male care for offspring is rare (5-10% of mammalian species), mainly observed in monogamous species and always associated with biparental care (Clutton-Brock, 1991; Lukas & Clutton-Brock, 2013). Moreover, paternal investment varies in terms of intensity and strategies from females, making it one of the most sexually dimorphic behaviour in mammals (see Royle et al., 2012). On a proximate scale, this dimorphism as well as other sexually dimorphic traits, was long thought to be a product of gonadal steroid hormones, acting to shape brain neural circuits (Arnold & Gorski, 1984; Breedlove et al., 1999). Over the past two decades, a sex chromosome perspective challenged the view of sexual dimorphism providing increasing evidence for the direct role of the sex chromosome complement (XX or XY) (Arnold, 2004; Gatewood et al., 2006; Mank, 2009; Ngun et al., 2011; Skuse, 2006; Rice, 1984). More precisely, genetically modified mice have allowed to dissociate individuals' gonadal and genotypic sex such as in the four core genotypes (FCG) model, where the *Sry* gene has been deleted and translocated on an autosome (XX and XY^- females, XX*Sry* and XY^-*Sry* males) (see Arnold, 2020). These advances have been crucial to decipher the direct role of both hormones and sex chromosomes on social behaviours (De Vries et al., 2002; Gatewood et al., 2006; Kopsida et al., 2013; Ngun et al., 2014; Skuse, 2006). However, very few have examined how sex chromosomes shape parental care and underlying brain neural circuits (Gatewood et al., 2006).

In rodents for instance, males tend to have more cell bodies and greater fiber densities of vasopressin expressing neurons, a neuropeptide involved in crouching over the pups or maternal aggression for instance, likely to result from an imbalance in X chromosomes

between the sexes (Crenshaw et al., 1992; De Vries & Miller, 1998; De Vries et al., 2002; Gatewood et al., 2006; Bosch & Neumann, 2012; Cox et al., 2015; Miller et al., 1989;Rood et al., 2008; Rood et al., 2013). Closely related to vasopressin, oxytocin is another substantial neuropeptide for parental care, well-known for its involvement in lactation, but also for enhancing pup retrieval and maternal aggression in females (Gimpl & Fahrenholz, 2001; Pedersen et al., 2006; Okabe et al., 2017; Scott et al., 2015; Bosch & Neumann, 2012). However, the impact of the sex chromosome complement on oxytocin in a parental context in the FCG or other mouse model has never been investigated. While the FCG has been a breakthrough in pointing out the value of the sex complement on parental behaviours, the common view of the system is far from complete considering the complexity of parenting behaviours as well as the various underlying neural pathways. Additionally, studies have only focused on genetically modified animals so far -which may have some limitations (e.g. absence of Sry in XY- females)-, where naturally occurring sex reversal systems subject to natural selection could bring insightful information especially in terms of brain and behaviours' evolution.

In natura, only few rodent species display atypical sex determination system with loss of the Y chromosome in males, or females carrying a Y chromosome (Saunders & Veyrunes, 2021). Among them, the African pygmy mouse, *Mus minutoides*, is peculiarly interesting as its evolution led to a third feminizing sex chromosome, X*, and three distinct female genotypes, XX, XX* and X*Y, while all males are XY (Veyrunes et al., 2010a). Sex-reversed X*Y females are fully fertile (Rahmoun et al., 2014), with an even higher reproductive success than XX and XX* females (Saunders et al., 2014). In addition, X*Y females are more aggressive towards intruders, display reduced anxiety, and have a greater bite force in comparison to XX and XX* females (Saunders et al., 2016; Ginot et al., 2017). These male-specific behaviours observed in X*Y individuals, which are not likely

to be testosterone dependant (Veyrunes et al., in prep), suggest that the Y chromosome masculinizes some neural circuits in the brain of *M. minutoides*. The highly contrasted phenotypes between female genotypes represent thus a tremendous opportunity to understand the neural bases of behaviours shaped by evolution. Furthermore, the dissociation of gonadal and genotypic sexes make of the African pygmy mouse a key species to investigate the effect of sex chromosomes on sex differences in brain and behaviours. In this study, we used an integrative approach to investigate the effect of genotypic sex on maternal care in *M. minutoides,* from behaviours to underlying neural circuits. We focused neural investigations on neurons expressing oxytocin and vasopressin in the paraventricular nucleus of the hypothalamus (PVN), a region and neuropeptides whose role in parental behaviours have been widely described. Since oxytocin neurons in the PVN are modulated by dopamine neurons in the anteroventral periventricular nucleus of the hypothalamus (AVPV) (Scott et al., 2015), we also investigated neurons expressing tyrosine hydroxylase (Th), the rate limiting enzyme for dopamine synthesis, in the AVPV.

Material & Methods

Animals identification and study environment

Behavioural experiments were conducted in accordance with European guidelines and with the approval of the French Ethical Committee on animal care and use (No. CEEA-LR-12170) in our laboratory colony (CECEMA facilities of Montpellier University) established from wild-caught animals in Caledon Nature Reserve (South Africa). Female genotype was identified whether by PCR of *Sry* using total genomic DNA extracted from tail tip biopsies with DNeasy blood and tissue kit (Qiagen) and following Veyrunes et al. (2010a) protocol or by karyotyping using bone marrow of yeast-stimulated individuals as previously described (Veyrunes et al., 2010b).

Behavioural assays

We tested primiparous females (i.e. first litter), between 2-10 days post-parturition (pp) for pup-directed care investigations and from birth to 14 days pp (before pup(s) start(s) to explore outside the shelter) for maternal care strategy. Tests were conducted during the day, between 10.00 am and 6.30 pm, by an experimenter blind to females' genotype. Each assay was filmed for subsequent analysis at the exception of maternal care strategy test, which was scored by punctual observations in the cage. When individuals were assessed in more than one assay, we conducted experiments sequentially by increasing magnitude of stress: we first tested females for pup retrieval, followed by mother-pup interactions and then, nest building, with a 15 hours gap at least between each test. Behavioural data were analysed blindly regarding females' genotype, using Observer v5.0.31 and Observer XT softwares (Noldus).

Pup retrieval experiments were carried out in home cage (31 x 21 x 21 cm). The father was removed along with the mother while pups were placed at the opposite corner of the shelter with the nest. An assay started once the female was put back under the shelter and recorded until at least one pup was retrieved. If no pup had been retrieved at 10 minutes, the assay was ended. A total of 101 females (31 XX, 35 XX* & 35 X*Y) were assessed on first approach latency and retrieval probability, each.

Mother-pup interactions experiments were carried out in a cage (38 x 26 x 24 cm) filled with individuals own bedding. We placed one pup at the centre of the cage only to account for litter size differences between females. We then placed the mother at a corner of the cage and recorded grooming, crouching and carrying behavioural responses during 10

minutes. We assessed 75 females (25 per genotype) on whether they displace/carry their pup, as well as duration of grooming, huddling and crouching behaviours.

Nesting test were performed early evening and nest were scored in the following morning after 12 hours of experimentation for 90 females (34 X*Y, 27 XX and 29 XX*). The father was removed from the cage as well as food, water, and all environmental enrichments except bedding. We added one nestlet (square of compressed cellulose) to provide nesting materials. Individuals received the same nestlet prior to experiment (post-parturition) for habituation. Nest scoring was assessed from 0 to 5 according to Gaskill *et al.* (2013) graduation: 0-cellulose unused and no nest, 1- no nest site, 2- flat nest, 3- slightly curved nest, 4- nest with walls, 5- fully enclosed nest. If a nest was built without using cellulose. We scored the nest accordingly to the graduation above but with more flexibility allowing intermediary scores as well (e.g. 3.5 or 4.5).

Parental care strategy, or whether mice display biparental care *versus* maternal care only was investigated for 105 females (29 XX, 37 X*Y and 39 XX*). We assessed strategies looking at fathers' involvement with pup(s), their position in the cage and possible physical injuries. We scored fathers' involvement gradually and increasingly, from 1 to 6: 1= father killed or removed for severe injuries, 2= chased, with aggression stigmata, 3= always in a different lodge, not tolerated in the shelter with the pup(s), 4= tolerated inside the shelter but in the opposite corner, 5= under the same shelter, next to the mother and 6= paternal care observed: huddling, carrying, crouching. Score measurements were repeated every 2-3 days during the first two weeks (except if the father was killed or removed i.e., score 1 or 2, referred to as high level of maternal aggression).

Immunohistochemistry

We sampled virgin individuals only as no experimentations leading to death can be made on mothers. Eighteen virgin mice (3-5/ genotype including males as a control group) were euthanised with isoflurane 4% anaesthesia followed by decapitation. Brains were collected in 4% PFA and stored for at least 48 hours at 4°C. Brains were sliced at 30 µm using a vibratome (Leica Microsystems) and coronal sections were collected into a PBS-filled container. Floating brain slices were then put in 4x3 well plates (4 wells per individual) and washed three times with Phosphate Buffer Saline (PBS) before staining. We sliced and stained the whole brain to account for any potential divergence from *M. musculus* in the brain anatomy (no data were available on the brain of *M. minutoides*). Brain slices were first incubated for 24h at 4°C or two hours at room temperature (RT) with a Goat coupled Fab anti-mouse IgG blocking buffer (1:1000, Abliance) diluted in PBS with 0.1% Triton 100X (PBS.T) and ~1% BSA to prevent non specific binding. Then, slices were incubated for 24-48h at 4°C with mouse anti-Neurophysin I (1:2000, Merck Millipore, marker for Oxytocin), rabbit anti-vasopressin (1:1000, Immunostar) and sheep anti-Th (1:1000, abcam) diluted in PBS.T and BSA, followed by secondary antibodies : Cy3 coupled Donkey anti-mouse IgG antibody (1:1000, Jackson Laboratories), Cy5 coupled Donkey anti-rabbit IgG (1:1000,Jackson Laboratories) and A488 coupled Donkey anti-sheep IgG (1:1000,Jackson Laboratories) diluted in PBS.T and BSA, for 24-48 hours at 4°C. Slices were finally incubated for 3-6 minutes with DAPI (1:10 000, Invitrogen) diluted in PBS and mounted on Polysine® Slides stored at 4°C until imaging.

Imaging and cell counts

Brain slices including the PVN and AVPV were identified based on the mouse brain atlas from Franklin and Paxinos (2001). Selected slices were processed with a scanner (Zeiss Axioscan) or confocal microscopy (Zeiss, LSM 780) and cell counts were done blindly and manually on imageJ, for entire nuclei. We also quantified neurons on few brain slices using

Imaris v9.8 software to compare with manual counts. We used it only for oxytocin as the software did not perform well for vasopressin neurons. Oxytocin and vasopressin cell counts as well as their distribution across the whole PVN nucleus were investigated to account for potential differences in localization between genotypes.

Statistical analysis

All statistical analyses were conducted on R v4.1.2 (R Core Team, 2021) using *lme4, stanarm, ordinal* and *multgee* packages. Pup retrieval was investigated with bayesian (generalized) linear mixed models, incorporating individuals' age as a random effect on the intercept. We fit our models to assess retrieval probability and visit latency using a log(x+1)-transformation to correct for a right-skewed distribution (many latencies at 600s). We ran four Markov chains of 2000 iterations each, removed the first 1000 (warm-up) and kept the second half to estimate our parameters. Both our models checked convergence recommendations ($\hat{R}$<1.1). Post-hoc comparisons were made using *emmeans* package to contrast each genotype. Point estimates reported per genotype are medians (back transformed from the log (x+1) scale) and median-based contrasts with 89% Highest Posterior Density intervals (HPD) excluding one were considered as truly different. Mother-pup interactions were also analysed with Bayesian (generalized) linear mixed models with individuals' age as a random effect and following the same steps as stated above. We log(x+1)-transformed duration responses to correct for a left-skewed distributions (many durations at 0s) and post-hoc comparisons were made to contrast genotypes. Nesting scores were analysed with a cumulative logit model (clm) and probabilities for nest quality per genotype were retrieved accordingly. XX was set as the reference group and we made multiple pairwise comparisons (*emmeans*) with adjusted p-value to assess each genotype effect. Parental care strategy was assessed with a population-averaged ordinal model to account for repeated measures and possible correlation between observations using

ordLORgee() from *multgee* package (Touloumis, 2015). Probabilities were then retrieved using a back-transformation of parameters estimation according to a cumulative logit link. Cell counts for immunostaining assays were analysed using the non parametric kruskal Wallis test for multiple group comparisons.

Results

Higher retrieval efficiency in X*Y females

During pup retrieval experiment, X*Y females approached their pup(s) significantly faster than XX and XX* females (Fig. 1a, Fig S1, table S1), with a median visit latency at 51.7s [33.6-73.8 89% HPD] versus 156.5s [94.5-223.3 89% HPD] for XX and 112s [71.5-158.6 89% HPD] for XX* females. Interestingly, 35.5 % of XX females did not visit their pup(s) while only 11.4% of X*Y and 17.1% of XX* females did not respond (table S2). To confirm that differences were not driven by the absence of response, we therefore reanalysed latencies only on females visiting their pup(s), and we found the same results (Fig S2). Additionally, X*Y females are significantly more likely to retrieve their pup(s) when compared to XX and XX* females, with an estimated retrieval probability of 0.87 [0.77, 0.95 89% HPD], while XX and XX* have a respective probability of 0.47 [0.32, 0.63 89% HPD] and 0.55 [0.42, 0.70 89% HPD] only (Fig. 1b, tableS1). Hence, X*Y females are more efficient at pup retrieval than XX and XX* females.

Poor nest quality by X*Y females

All females showed nesting behaviours as no nest score below 3 was recorded. However, X*Y females are more likely to build poor nest quality in contrast to both XX (Fig. 1c, z= -3.09, p=0.006) and XX* females (z=-4.61, p< 0.001). They have a probability of 0.35 (highest probability) for building a slightly curved nest (i.e. score 3) while they have a 0.17 probability, for making a fully enclosed nest (i.e. score 5). On the contrary, XX and

especially XX* individuals are more likely to build high quality nests with 0.47 and 0.71 probabilities respectively, for a fully enclosed nest. This also indicates a relatively high consistency in nest-building behaviour for XX* females. Though XX* have a higher probability than XX individuals for building high quality nest, they show similar behavioural trends (Fig 1c, z= -1.82, p= 0.16). Interestingly, most X*Y females did not use the nestlet to build a nest (88.2 %) in contrast to 48.1 % for XX and 41.4% for XX*. We therefore tested for a relationship between nest quality and nestlet usage (i.e. disinterest for the nestlet or poor learning) in X*Y females and did not find any correlation (χ^2 =10.05, p=0.19). X*Y females are thus less efficient at nest building when compared to XX and XX* females.

Genotypic sex influences female parental strategy

X*Y females are in average more likely to show maternal aggression (i.e. score 1) than XX and XX* females, which inversely, are more likely to display biparental care (Fig 1d, X*Y vs XX, z=-2.81, p=0.005; X*Y vs XX*, z=-2.87, p=0.004). Indeed, X*Y females have a highest probability at 0.33 to severely aggress or kill males (i.e. score 1) in contrast to a 0.19 probability to display biparental care (i.e. score 6). Inversely, XX and XX* females have highest probabilities at 0.46 and 0.43 respectively for biparental care in comparison to 0.12 and 0.13 probabilities for maternal aggression. Strikingly, XX and XX* females curves almost overlay with quite similar probabilities in each category highlighting identical parental care strategy in XX and XX* females.

Genotypic sex does not impact mother-pup interactions

Most females interacted with their pup and there were no significant differences in interaction durations nor probability (table S3, Fig S3-4). X*Y, XX and XX* females spend in median 7.9s [3.07-13.2 89% HPD], 11.2s [4.65-18.8 89% HPD] and 15.9s [7.03-25.8 89% HPD] crouching over the pup, 7.17s [3.48-11.36 89% HPD], 4.96s [2.26-7.97 89% HPD] and 7.30s [3.58-11.64 89% HPD] huddling with the pup, 3.18s [1.36-4.99 89%

HPD], 4.87s [8.49-7.46 89% HPD] and 5.79s [2.89-8.70 89% HPD] grooming the pup, respectively. In addition, they have respective probabilities of 0.68 [0.42-0.9 89% HPD], 0.76 [0.5-0.93 89% HPD] and 0.67 [0.4-0.87 89% HPD] to displace their pup.

Hypothalamic neural circuits of maternal care

We did not find evidence for difference in the global neuroanatomy of *M.minutoides* PVN in comparison to *M.musculus*. Vasopressin is expressed in both parvo- and magnocellular neurons, mostly in intermediate regions of the PVN where it is expressed dorsolaterally (Fig S5, S7-S10). Oxytocin is mostly expressed in magnocellular neurons and homogeneously across the nucleus (Fig S6-S10). We did not have enough power to statistically compare oxytocin and vasopressin neuron distributions across genotypes but there were no overall striking differences (Fig S5-S6), with high variabilities in count data due to inter-individual variability and small sample sizes. In addition, there were no differences in oxytocin neurons number between genotypes (Fig 2a,c, $\chi^2 = 0.89$, $p= 0.83$), albeit a slight tendency for a greater number in males (Fig 2-S6). We did not find differences in the number of vasopressin expressing neurons either (Fig 2b,c, $\chi^2=0.82$, $p= 0.84$) but there is a tendency for lower number in X*Y females driven mostly by the lower number of vasopressin neurons in anterior regions (Fig S5). Finally, there were no differences in the neuroanatomy of the AVPV in comparison to *M.musculus.* We weighted the number of Th expressing neurons by the area of the nucleus and did not find differences in the density of Th neurons between genotypes ($\chi^2=2$, $p= 0.57$), though a pronounced tendency for a greater density in X*Y females (Fig 3).

Discussion

The African pygmy mouse is a species with a naturally occurring sex reversal system, involving a dissociation between phenotypic and genotypic sexes in X*Y females. This

fascinating system represents a great opportunity to shed light on the impact of the sex chromosomes on sexually dimorphic behaviours such as parental care. By focusing on maternal phenotypes specifically, we show that the sex reversal in *M. minutoides* influences pup retrieval, maternal aggression, nest building behaviours and may also impact the maternal dopaminergic circuit in the AVPV nucleus. Our study confirms and further extends evidence on the influence of the sex chromosome complement on parental behaviours and brings promising prospects, for its impact on the neurobiology of parenting.

X*Y females are more efficient at pup retrieval than both XX and XX* females. They first approach their pup faster and show consistent retrieval, while XX and XX* show delayed latencies to first approach and retrieve their pup about one out of two times. Moreover, in a reproductive context, X*Y mothers are highly aggressive towards males, whereas XX and XX* females share parental care with males. Previous results showed that non-reproductive X*Y females are more aggressive than other female genotypes towards intruder males (Saunders et al., 2016). Here, we show this enhanced aggressiveness increases further post-parturition, even towards the pup's own father (as male partners were killed or needed to be removed for 43.2% of X*Y females (i.e., 16 out of 37)), suggesting that X*Y females are more likely to be solitary mothers. Furthermore, X*Y females build poor quality nest (slightly curved), while XX and especially XX* females perform better with fully enclosed nests. It was surprising that almost all X*Y females did not use the nestlet, and the lack of correlation between nestlet usage and nest quality strongly suggest a disinterest for the nestlet in X*Y females. Because nest building is an essential component of maternal care especially in altricial species, to protect the young from potential threats for instance, this would thus imply maladaptation in X*Y females. However, we show here that X*Y females can assure pups protection through agonistic

behaviours (i.e. maternal aggression), which might thus make the nest quality less essential for these females. Hence, the divergent behavioural patterns between X*Y and XX / XX* females reflect two distinct maternal strategies shaped by the sex chromosome complement in *M. minutoides*. Although there is an unequivocal effect of the sex chromosome complement on some maternal behaviours, there were no differences in mother-pup interactions suggesting that it does not modulate all component of maternal care.

It is very striking that X*Y females display quite consistently divergent maternal behaviours: they show a greater retrieval efficiency, high level of maternal aggression and poor nesting skills, all of which differ from XX and XX* females. While the two formers correlate with a feminizing X* chromosome, the latter suggests masculinization with impaired nesting behaviour as found in males (data not shown; Topilko et al., 2022) due to the Y chromosome and Sry expression in X*Y females (Gatewood et al., 2006, Rahmoun et al., 2014). Interestingly, this pattern of both masculinized and hyper-feminized traits was already present in previous studies on the consequences of the sex reversal in *M. minutoides*. For instance, X*Y females are not only fertile, they also have a higher reproductive success than both XX and XX*: they breed earlier, have bigger litter sizes and therefore produce more offspring in average over a lifetime period (Rahmoun et al., 2014; Saunders et al., 2014). Furthermore, X*Y females are more aggressive than XX and XX* females and with a greater bite strength which are male specific traits (Saunders et al., 2016; Ginot et al., 2017). All things considered, the constant divergence between X*Y on one side and XX and XX* females on the other side highlights that the association of a feminizing X* and a male-specific Y chromosome has given birth to a third sexual phenotype in the African pygmy mouse.

Interestingly, our results are opposite to previous findings on the FCG in which sex reversed XY- females displayed similar nesting behaviours to XX females but impaired retrieval capabilities: they retrieve less pups and belatedly when compared to XX females (Gatewood et al., 2006). The authors suggested that either imbalance in X-linked genes due to the presence of a single X chromosome, the Y chromosome or the *Sry* gene was sufficient to alter maternal phenotypes. In our case, we show that X*Y females perform better at pup retrieval even though they have a Y chromosome and express *Sry* in their brain (Rahmoun et al., 2014). Therefore, either Y-linked genes including *Sry* act distinctly on neural pathways between FCG mice and a wild mouse species, or it is an X* chromosome effect. Since XX and XX* behave similarly, an X* chromosome effect would imply maternal X*-biased chromosome inactivation (X*CI) in the brain of XX* females, or parental imprinting of X-linked genes (Gregg et al., 2010; Raefski & O'Neil, 2005), with preferential expression of the paternal X alleles. There is no evidence for skewed X*CI in *M. minutoides* based on cytological investigations of embryos fibroblast cell cultures (Veryunes & Perez, 2018), but next generation sequencing technologies with a special focus on allele-specific expression in the brain should help clarify hypotheses. Nonetheless, contrary to expectations based on the FCG, the sex reversal in the African pygmy mouse does not seem to alter maternal care quality. This discrepancy further illustrates the need to consider not only genetically modified laboratory mice but also non-model species with alternative phenotypes shaped by natural selection -such as observed in *M. minutoides*-, to better understand the genetic and neural bases of behaviours.

In mammals, oxytocin and vasopressin are crucial neuropeptides that intervene in the initiation and maintenance of parental care (Zilkha et al., 2017). In rodents, oxytocin expression in the PVN induces pup retrieval and maternal aggression (Neumann et al., 2005 ; Pedersen et al., 2006; Scott et al., 2015; Okabe et al., 2017; Bosch & Neumann, 2012) while vasopressin, which can also mediate maternal aggression, has been shown to

inhibit nest building (Bendesky et al., 2017; Bosch & Neumann, 2012). Moreover, vasopressin has been shown to be influenced by X chromosome number, with greater expression in individuals with a single X chromosome (De Vries et al., 2002; Gatewood et al., 2006, Cox et al., 2015). Hence, oxytocin and vasopressin were prime candidates to explain the maternal behaviours in *M. minutoides*, expecting a greater number of vasopressin and oxytocin neurons in the PVN of X*Y females in comparison to XX and XX* females. However, we did not find such differences between females nor with males, emphasized by high variability in count data (i.e. indicative of low power; Fig 2a,b). This suggests that there are no differences in oxytocinergic nor vasopressinergic systems global anatomy in the PVN, which is consistent with gene expression results in virgin mice with Turner (XO females) and Klinefelter syndrome (XXY males) (Cox et al., 2015). On the other hand, the lack of differences may not be as surprising using cell counts only and the functionality of the neuroendocrine networks as well as the hormonal output should therefore be tested (e.g. quantification of gene and protein expression in the PVN, blood hormonal levels measured over time) (Cox et al., 2015; Scott et al., 2015; Bendesky et al., 2017).

Another interesting brain region to look at was the AVPV. It has been shown that neurons expressing Tyrosine hydroxylase (Th) in the AVPV ($AVPV^{Th}$) stimulate oxytocin expressing neurons in the PVN and consequently pup retrieval in females (Scott et al., 2015). Moreover, these neurons are sexually dimorphic with a greater number in females than males already in virgin individuals (Semaan & Kauffman, 2010; Scott et al., 2015). Our results do not support the sexual dimorphism described in *M.musculus*, however we show a tendency for a greater density of Th neurons in X*Y females, which is consistent with pup retrieval results. Though not significant, it brings new perspectives on the link between sex chromosomes and the dopaminergic system as well as between dopamine and parenting.

Finally, it is noteworthy that parenting is a complex behaviour that is regulated by diverse neural pathways and neuromodulators. In rats for instance, it has been shown that overexpression of the neuropeptide Y, which is sexually dimorphic, correlates with impaired nesting behaviours (Nahvi & Sabban, 2020 ; Corder et al., 2020). It is thus not unlikely that the sex chromosome complements in *M. minutoides* act on various neural circuits to shape maternal behaviours, and future studies should integrate other brain regions and neuromodulators, to help understand the neural basis of maternal strategies. Because we work on a wild species with a precious status, we are facing technical and interpretation limits due to small sample sizes and virgin individuals while parental neural circuits are known to evolve with sexual experience (Lopatina et al., 2011; Scott et al., 2015; Okabe et al., 2017; Zilkha, Scott & Kimchi, 2017). Moreover, we are limited by technical approaches as we cannot inject viruses to evaluate neuronal projections, undertake optogenetic stimulation nor ablation of neuronal population to establish a causal link between brain nucleus, peptides and behaviours. Nonetheless, primary investigations on the neuroanatomy are essential to describe and further understand the evolution of the brain and behaviours as illustrated here in a wild species with highly contrasted phenotypes.

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

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Figures

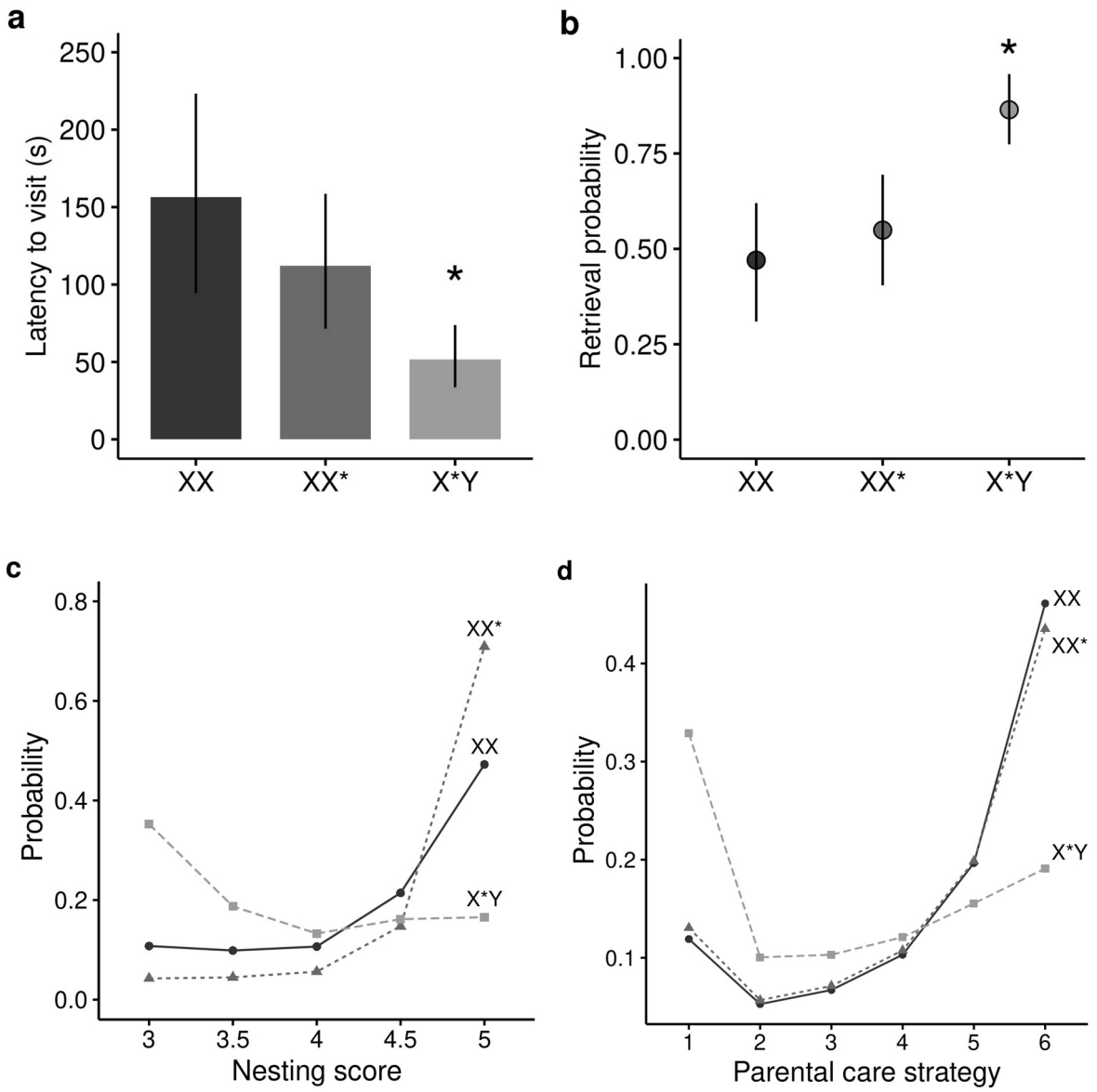

Figure 1. The sex chromosome complement shapes maternal behaviours in the African pygmy mouse. **a,b**, Pup retrieval. Estimates of first visit latency and retrieval probability per genotype. Values are medians with 89% HPD intervals. Latency to first visit and probabilities are retrieved from a back-transformation 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 as well as to 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=0.002) and XX* females (p<.0001). n_{XX}=27 , n_{XX*}=29, n_{X*Y}=34 **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.

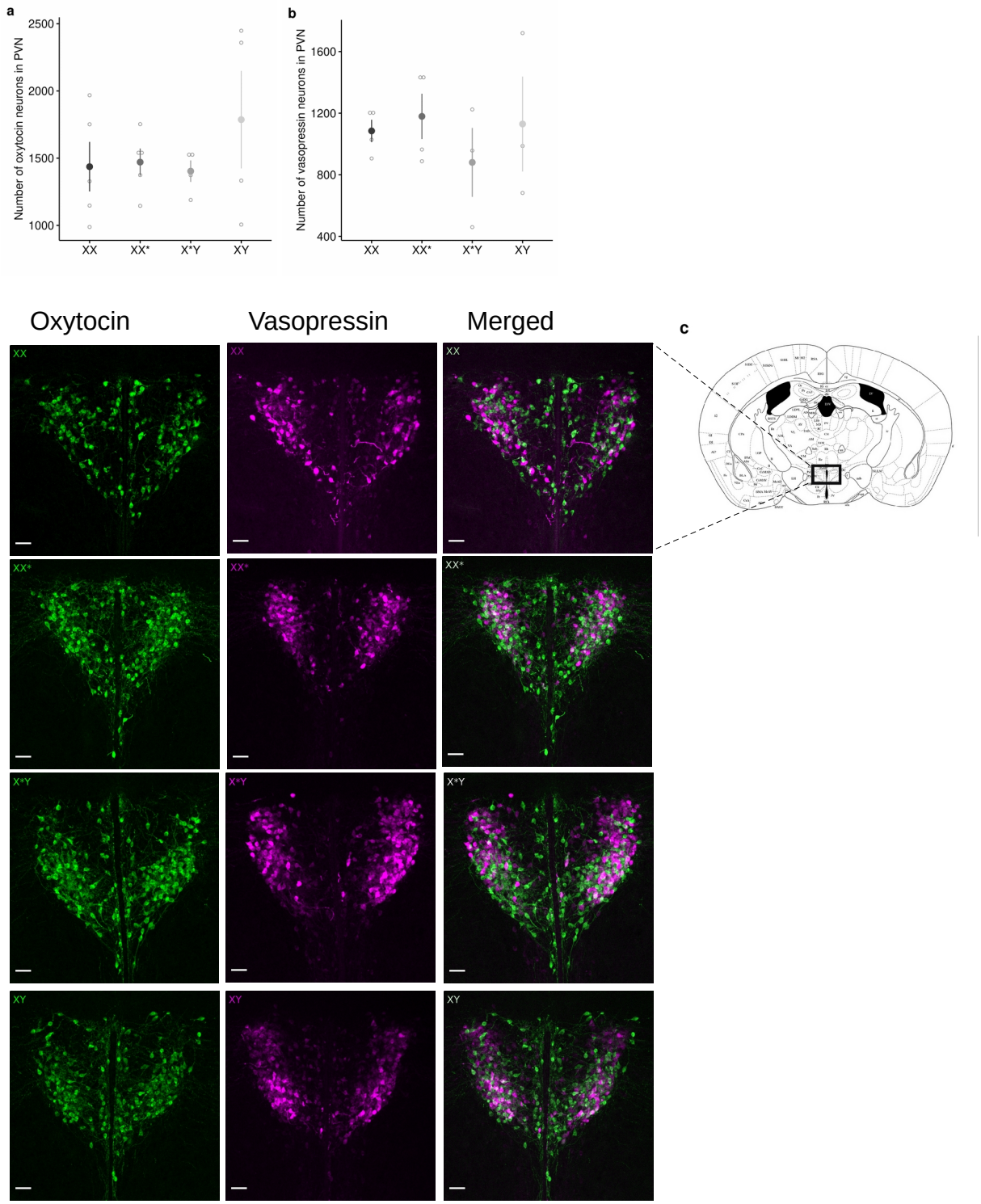

Figure 2. No genotypic sex effect on oxytocin and vasopressin neurons' number in the PVN of virgin individuals. **a,b**, Total number of oxytocin and vasopressin expressing neurons in the PVN of virgin XX, XX*, X*Y females and XY males. Oxytocin : n_{XX}=5 , n_{XX*}=5, n_{X*Y}=4, n_{XY}=4. Vasopressin : n_{XX}=4 , n_{XX*}=4, n_{X*Y}=3, n_{XY}=3. Data are means ± 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, 2001) . Scale bars 50 µm.

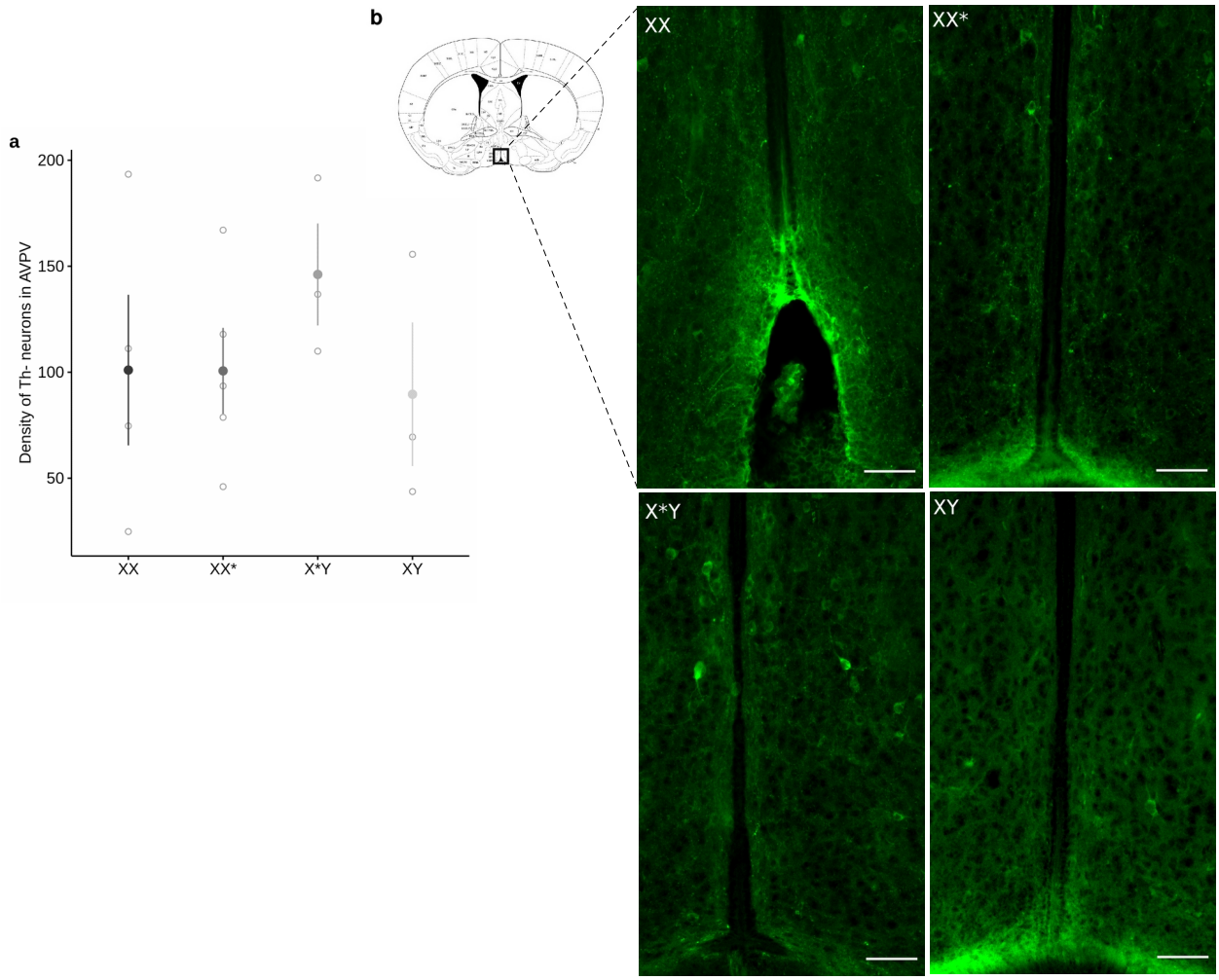

Figure 3. Tendency for greater Th neuron density in the AVPV of X*Y females. **a,** Density of Th- expressing neurons in PVN of virgin XX, XX*, X*Y females and XY males: number of total Th neurons weighted by the sum of the area (mm2). n_{XX}=4 , n_{XX*}=5, n_{X*Y}=3, n_{XY}=3. Data are means ± s.e.m 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, 2001) . Scale bars 50 µm.