Dear recommender,

Please find below are responses to the referees.

Best regards,

Responses to reviewers

Reviewer 1
Reviewed by Marion Anne-Lise Picard, 26 May 2022 09:12

MINOR SUGGESTIONS:

1. The only important concern I could have is that their model do not allow them to perform the neurobiology assays on mothers, but on virgin females instead. Nonetheless, they adequately discuss this limitation (l390-l406) and their arguments are valuables. Even if I understand the reasoning behind the formulation "the neural basis of maternal behaviours", they maybe could find an alternative which better reflects the "potentiality"?

Yes, we agree that referring to the potentiality is more appropriate. We rectified it throughout the manuscript.

2. Sup.Fig. S5-S10: Why not comparing the "oxytocin neurons distribution" (S6) in the same way as vasopressin neurons (S5)? (It seems that at least that the regions #1 and #5 are also characterised by a tendancy for lower number of oxytocin neurons, aren't they?)

Besides, did the autor studied the distribution of cell on left vs right side??
Indeed, there are growing evidences that the hypothalamus is functionally lateralized (Kiss et al. Brain Science, 2020 for review : https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7349050/). Such analysis may allow them to refine the signal?

You are right, there is a tendency for lower number of oxytocin neurons in region #1 and #5. We added this point below Fig. S6.

It is indeed a possibility that the PVN show spacial variations between left and right hemispheres. Unfortunately, we do not have this information due to our brain slice protocol, as slices were dispatched in a plate altogether, they were not directly put on a slide, so we cannot track the laterality of the brain reconstruction and cannot determine the right and left hemispheres.

3. Finally, did the authors previously assessed the level of gonadal hormones in the different female genotypes? Are they similar?

We did, the data are available on BioRxiv. We investigated corticosterone, testosterone and estradiol. There is no striking differences in hormones except for corticosterone, X* Y females have a male-like level of basal corticosterone.

VERY MINOR SUGGESTIONS:
-Fig2 : make slightly bigger panels A & B ?
-Even if it is well explained in the text, maybe the authors could had a small chart of the studied neural circuit?

Yes, the panel were small, we increased the size and added an illustration of neural circuits and predictions in the neuroanatomy of oxytocinergic, vasopressinergic and dopaminergic system.

SOME TYPOS:
l26 (keywords) : natural sex reversal and not "sex reversal, natural," ?
l106 : remove the space before "Behavioural"
l214 : remove space before "Higher"
l352 : change the ref. (Veryunes & Perez, 2018) for (Veyrunes & Perez, 2018)
l622 & 654: remove the space after "2001)"

All of these have been rectified, thank you for pointing them out.

Reviewer 2

Please provide much more detail in the methods. Here is a partial, but not exhaustive list:

1. How many generations were the mice bred in the lab after being caught in the wild?
2. Many more details on the husbandry of the animals is necessary. For example: what do you feed them and how often, in what cages and what dimensions are they kept, is the room temperature controlled and what temperature, light cycle conditions, etc.
3. Are X* Y sisters and littermated of XX* ? Otherwise, there is a chance that differences in behavior between the genotypes are related to other genetic differences between X*Y and XX* (and XX) due to population structure (in the wild or in the lab) rather than to the genotype of the sex chromosomes themselves. Please describe how the individuals used in the experiments were generated.

We added the information related to these 3 points lines 107-117. As for X* Y females, you are absolutely right ; however here, X*Y and XX* females are both littermated of X*Y or XX* females, while XX are littermated of XX or XX* females. In consequence, the three female genotypes can potentially be sisters. We thus eliminate population structure or maternal effects.

4. Lines 119-120: How many pups are placed in the cage for tests of pup retrieval? And how was this normalized across females with litters of different sizes?

We used the entire litter which indeed varies between females, which is why we focused only on the first pup retrieved. It is described lines 131-134

5. The dimensions of the cages are not clear. Which one is the height?

It is standard nomenclature for dimensions so length x width x height

6. Line 136: More details on the nestlet. What exactly is this compressed cellulose? What brand? What dimensions and weight?

We added the informations lines 149-150(Serlab, D00009)

7. Line 140-141: I don't understand "If a nest was built without using cellulose". What else could it have been built with if you only provided cellulose as nesting material?

They can also use bedding, we clarified it.

8. The parental care strategy measurement is not clear due to insufficient details. For example, was this done in the home cage or a new cage? How long is each observation? Do you average all observations? Was the male ever separated from the female and added back for this assay or is this just observations in the cage where the female and male reproduced, without disturbing it. Also, regarding the assay and its interpretation, this measurement reflects the behavior of the male as much as that of the female. Are you confident you are measuring the "strategy" of the female rather than the interest of the male in the pups because random males are placed with females of all three genotypes?

More informations are provided in the specific section. All observations (every two-three days from day of parturition of the first litter to day 14) were made in home cage, so where male and female reproduce and without manipulation.

We talk about parental care strategy and not only female strategy in this experiment. Nevertheless, since the score attributed to the males relies on female behaviours : whether the female chases him (once the male goes nearby the female, she instantaneously attacks him) or includes him in the nest, we are confident that parental strategy relies mainly on the strategy of the female (it is the female behaviour towards the male that impacts the father's involvement). Even in bi-parental care, it depends mainly (only ?) of the choice of the female to allow the male to provide care. Therefore, we do have different strategies between females as the genotype is the only varying factor in these experiments.

It is an average population model because of repeated measure over time but not for all categories (we did not repeated measure when the score was 1 because we had to remove the father or he was already killed). Therefore we cannot make a standard ordinal model. In this model, we include all the data with information about repeated measures per individual and the corresponding score to estimate the average probability of falling into a class or under (see Touloumis et al, 2015 for further model explanation).

Other comments

9. Lines 156-157 and 401: I didn't understand why you cannot inject viruses into the brains

of these mice nor euthanize mothers to perform analyses of their brains or other experiments.

**We cannot for multiple reasons : probably the first one is our own personnal ethics about animal welfare, we do not feel comfortable about performing these approaches.
Second, It's a wild species that is hard to reproduce in laboratory and we cannot take the risk to kill mothers and the corresponding litters. Third, all experimentations such as optogenetic studies are performed on lab mouse, this system is not fit for pygmy mice that weight on average 8g with a size of 6cm. As for virus, similarly, it is based on lab mice and rats, we need stereotaxic coordinates of the brain which we do not have in our mice. It is the first study that investigates the brain of this wild mouse species.**

10. Lines 10-11: I think the authors mean that the sexually dimorphic nature of parental care is largely explained by differences in gonadal hormones between the sexes, but this is not clear.

Yes, we clarified this point in the abstract.

11. Lines 23-24 and 336-337: I'm not sure why the unique maternal care behavior of X*Y females is being labeled as a "third sexual phenotype". What is a "sexual phenotype"? And wouldn't the results of the authors actually be consistent with maternal behavior in these mice not being a "sexual phenotype" since all three of XX, XX* and XY* are females (gonadally and in terms of reproductive anatomy) so their (gonadal) sex doesn't differ, only their behavior?

It is not only the maternal behaviours that makes us talk about a third sexual phenotype. It is the combination of our present and previous results: agressive and anxiety related behaviours, life-history traits, bite strength etc.. (Saunders et al. 2014, 2016 ; Ginot et al. 2017). When we talk about sexual phenotype we need to distinguish between gonadal sex and other sexual traits such as gender (sexual characteristics/ differentiation of the brain). Gonadal sex is indeed either testis or ovaries ; but when it comes to other phenotypes : it is not dichotomous with either stereotypic male and stereotypic females. Sex is multiscale and gender for instance is not binary. The consistent divergence of patterns between the X*Y females and the other females and some similar traits between males and X*Y females allow us to talk about another female phenotype, therefore a third sexual phenotype.

12. Lines 30-31: Missing a comma after females. Also, "invest more notably as a primary result of an obligatory lactation" is not clear. A physiological result? An evolutionary consequence? There are mammals such as marmosets in which most of the parental care (except for lactation) is performed by males. Also, in birds, females often do more parental care than males, even though there's no lactation. Thus, higher maternal than paternal care is related to higher investment by females than males in offspring (including in making eggs and in preganancy), not necessarily a consequence of lactation itself.

We only talk about mammals here and lactation in mammals makes maternal care obligatory, so there already is a bias. But yes, you are right lactation does not necessarily mean female-skewed parental care. Nevertheless, it is rare that fathers undertake more care than females

as found in Marmosets. Usually, females invest more and they have no "choice" to invest because of lactation otherwise the offspring will die.

13. References such as Rice, 1984 are missing from the reference list.

It was present but misplaced, we rectified it.

14. Lines 75-78: I'm not convinced about calling high aggression, reduced anxiety and greater bite force as "male-specific behaviors". I wouldn't, for example, call elevated height as a male-specific human trait, even though on average human males are taller than females. Please consider rephrasing.

The reduced anxiety behaviours and higher bite force of X*Y females are comparable to males but different from the other female genotypes (see Saunders et al., 2016, Ginot et al., 2017), therefore they show masculinized patterns. Concerning aggressive behaviours, even if they are observed in both sexes, these traits are mainly observed in males, they are sexually dimorphic in many diverse taxa. Furthermore, gonadal hormones and notably testosterone act perinatally to induce masculinization of neural pathways involved in sexually dimorphic behaviours including aggression.

Comments 15, 19 & 20:
15. Lines 78-79: I don't think there's enough evidence to say that the Y chromosome masculinizes neural circuits in X*Y M. minutoides. It could be a lack of a second X chromosome or X* masculinizing them when there is a Y chromosome (i.e. an epistatic effect).

Indeed, it is an hypothesis based on the study of Gatewood et al., 2006 where they made the assumptions that Sry was a strong candidate for nest building inhibition as sex reversed females XY without Sry built good quality nest. You are absolutely right though, it can be an X dosage imbalance females being heterogametic even if results of Gatewood et al, strongly suggest that nest building is not impacted by dosage imbalance.

19. Lines 323-327: I don't understand this very long and convoluted sentence. Also, there is no evidence from this study that "masculinization" of X*Y females is caused by Sry. See comment 15.

We rectified the sentence and yes, you are right, it was an hypothesis based on previous findings (Gatewood et al., 2006), we clarified it.

20. Lines 329-332: Related to point 14 above, how is this evidence of masculinization or of a "hyperfeminine" trait? I would consider rephrasing these sections.

We changed "hyperfeminized" to 'feminized'. We agree it is more appropriate, we used hyper-feminized because they have a higher reproductive success than XX and XX* females : higher ovulation rate, greater litter size, precocious sexual maturity but considering maternal behaviours, it is not relevant. As for masculinization we talk about masculinized trait because the pattern observerd in X* Y females is similar to that of males.

16. Line 245: “on average” instead of “in average”.

Indeed, it is now changed.

17. Lines 278-279 and 384-385: Because you did not directly compare the neuroanatomy of AVPV between M. minutoides and M. musculus, the conclusion that “there were no differences in the neuronatomy of the AVPV in comparison to M. musculus” is a bit of a stretch. Please rephrase.

The neural structures, such as the paraventricular nucleus (same shape and neuronal populations ; expression/ distribution of oxytocin and vasopressin in parvocellular and magnocellular neurons) are overall similar between musculus and minutoides which we explain right after lines 279-282. We added references lines 284-285 and lines 297-300.

18. Lines 302-303: No direct comparison of aggressiveness was done between pre and post parturition, so this conclusion is not warranted.

Increases was indeed not appropriate, we changed it.

21. Lines 347-350: Related to point 15, this could also be due to epistasis between X* and Y?

It is a very good question and we cannot rule out an epistasis interaction, we included it in the X* chromosome effect but we clarified it along with the hypotheses on the rôle of each sex chromosome in the paragraph (line 354-401).

For instance and on that point, assuming we confirm the pattern of Th between females as we found also on RT-qPCR (see comment reviewer 3), one could hypothesize an epistasis interaction between the X* and Y on the dopaminergic system :1- we know that individuals carrying a single X chromosome have usually more dopamine, 2- Sry positively modulate dopamine through Th and 3- The AVPV (based on males *musculus*) is a rare region of the brain where Th is greater in females.
One could therefore assume an adaptive positive regulation of X* in interaction with Sry on the Y chromosome.

22. Line 397: What is a species with “precious” status?

Thank you for this comment, indeed, precious is not relevant here. We meant that some individuals are rare, it can be hard to breed them especially XX females. We changed it

Reviewer 3

Title: Clearly reflects article’s contents.

Abstract: Concise and presents main findings. The dopaminergic results, however, I think are too limited

(looking at data spread and sample size) to be interpreted as “likely” impacted by genotypic sex. More on
this in the Results section.

Introduction: Research motivation and questions are well described with some exceptions. Regarding
summarizing relevant prior work, I suggest making it explicitly clear which sex hormones have been
compared between the genotypic sexes in M. minutoides specifically. Otherwise, it still seems open-ended as to whether the behavioral results are due to hormonal or genetic differences.

This is an interesting comment indeed and we actually do not exclude a potential effect of hormones in the introduction. We included more informations about hormones in the discussion section lines 384-387. However since these are all females (i.e. same gonads), differences in behaviours arise at the very basis from differences in genotype : it can be sex-linked genes directly, genes under the influence of sex linked genes and X dosage, or interactions between sex chromosomes and hormones. In any case though, the prior basis for differences in behaviours, as long as the genotype is the only varying variable, are related to the sex chromosome complement.

Also, while the
functional relationships between vasopressin and oxytocin and behavior are described, it is not abundantly clear what expectations are regarding cell population numbers and the M. minutoides genotypic sexes.

Yes, you are right and we corrected it in the manuscript :lines 96-99 as well as with an illustration (Figure 1).

Materials and methods: There are several details that would improve the reproducibility of this work.

- Please provide time of experiments relative to light cycle (e.g. Zeitgeiber time).
- Please mention which, if any, criteria were used in pairing males and females. Also, please include female age at pairing and testing.

Thank you for these two points, we added the information line 105-109

-Can you provide at average period between the “punctual observations” or maternal care strategy?

It was written line 161-163 but we clarified the informations so it will be more clear (lines 161-163°

- Please mention what type of bedding was used during the nesting test. The bedding material would affect what kind of nest a mouse could build without using the cellulose nestlet.

You are right, it can influence however all females have the same bedding, therefore it won't change differences in nesting behaviours. We also show that X*Y females that have an elaborated nest mostly used the bedding instead of the nestlet : lines 248-250.

- In the statistical analysis section, the following sentence seems to be missing a reference: “Both our models checked convergence recommendations ().”

Thank you for the comment, it is not a reference under the brackets but a formula : R-hat <1.1, which for a reason I ignore, was removed.

Results: The behavioral results seem sound. The histological studies are quite limited in statistical power,
which the authors openly state. While I can sympathize with the challenges of working with a study species that harder to procure and/or more variable than inbred strains, these data and provided analyses all fall below my threshold for identifying any “tendencies.” I think the only sound conclusion to
be made is that “there were no overall striking differences.”

You are right, small sample size is really challenging especially when it comes to interprete data, we clearly have a lack of statistical power which is why any interpretation is limited and should be limited. I definitely agree with you that the combination of small sample size and the spread of the data here, especially for XX and XX* females, question the legitimacy to interpretate it as a true tendency. Graphically speaking, it is still however a tendency and we also find the same tendencies for greater expression of Th in the whole brain of X*Y females (rt-qPCR and RNAseq) with greater sample sizes than here (10-13 individuals). We discuss these unpublished data in the discussion, but we did not included them in the study itself as it is a part of a different study. Moreover, there is a tight link between Sry and Th which have been shown in mice that could support this tendency.

Discussion:

- In the section discussing chromosomal contributions to the X* Y phenotype, both the X* and the Y are discussed as potential sources of differences between X* Y females and the other female genotypes. Could X-inactivation escapees also play a role in the difference between X* Y females and the other female genotypes?

In this section we were confronting FCG results with ours especially pup retrieval in X* Y females while predictions related to the FCG mice would have suggested impaired retrieval. However, it is a very good point, looking at differences between XX/XX* and X* Y females, it is a possibility that imbalance in X-linked genes including X escapees could influence differences in behaviours. We clarified this section with all different hypothesis about the relative contribution of the sex chromosomes including epistasis interactions and X dosage.

- I think the discussion of oxytocin and vasopressin could be made clearer regarding relationships between anatomy, gene expression, and behavior. Do the authors expect that, given the lack of striking difference in cells numbers, that there would also be a lack of gene expression/peptide difference? The authors also mention earlier that reproductive status can influence these systems; I would like to see that discussed here to contextualize their results.

Indeed, we clarified this section. For the reproductive status, we did discussed it later but we moved this part in the discussion lines 421-424.

Figures:

- The way the figures printed out for me, the axes labels for the Figure 2 and Figure 3 plots are very small. The genotype labels over brain images in Figure 2 are also very small.
- Figure S4 is missing “c” panel.
- Figures S8-10. These images appear to come a single, representative female of each genotype. That should be made clear in the figure legend. Also, only “a” is in the actual figures, “b” through “e” are only mentioned in the figure legend.

Thank you for these comments , we made the change for the label sizes. As for « a » « b » « c » etc panels ; they were present but as I sent a doc file while I work on a linux machine, there is always rearrangements in files, I apologize for that. I will include the suppl data as a pdf so it won’t change the configuration.

References:

Seem fine, just need to address missing reference in Statistical Analysis section.

See comment above : not a reference but a missing formula.