

The neural mechanisms supporting the rise and fall of maternal aggression

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 1:

Reviewer comments:

Referee #1

(Remarks to the Author)

Comments on Yamaguchi et al. "The Neural Mechanisms Supporting the Rise and Fall of Maternal Aggression"

In this work, Yamaguchi et al. identify a two-tier neural mechanism that switches maternal aggression on and off. While it has long been known that mothers exhibit aggression towards intruders, the circuit basis for how this aggression is regulated remained unclear. Yamaguchi and colleagues uncover a neural pathway (PA → VMHvl) sufficient to trigger maternal aggression and identify synaptic and biophysical changes in PA and VMHvl that correlate with aggressive states. Moreover, they found that PA neurons projecting to VMHvl do not exhibit altered excitability in lactating mothers, suggesting an additional mechanism that enhances PA neuron output. They discovered that oxytocin signaling plays a crucial role in this process. Therefore, the rise of maternal aggression involves two mechanisms: first, biophysical changes in VMHvl neurons and changes in PA → VMHvl connectivity, and second, increased sensitivity to oxytocin signaling in PA neurons.

The major strengths of this work are threefold. First, the authors not only identify the critical roles of PA_Esr1 and VMHvl_Npy2r neurons in maternal aggression but also demonstrate that the monosynaptic connection between these neurons mediates this aggression. Importantly, they show that this pathway is specific to aggression and not involved in mating. Second, they combine in vivo and ex vivo analyses to pinpoint precise synaptic and biophysical changes across different estrous states and in lactating mothers. Third, through gain- and loss-of-function experiments, they demonstrate the key role of oxytocin in controlling this behavior.

Overall, this work represents a major milestone in understanding the circuit basis of maternal aggression. Several points could enhance the study's impact.

First, the manuscript appears to be written primarily for specialists in the field of mammalian social behaviors. This is a missed opportunity. To reach a wider audience, the authors could include a more general introduction and discussion, for example, on sexually dimorphic regulation of behavior and neuropeptide modulation of behaviors.

Second, while the authors convincingly show that the neural basis of aggression differs between lactating females and males, they have not demonstrated the exact link between sex differences and the observed phenomena. For example, what is the molecular basis of Oxt induction in mothers? The authors suggest that "The synaptic and cellular changes, likely induced by the cocktail of sex hormone surges during pregnancy and parturition, primed the aggression circuit to be ready to engage," but directly testing this hypothesis would significantly enhance the study's impact.

Third, several minor points could be clarified:

- Figure 1j: Please indicate where VMHvl is in this panel. It appears that most of the green signals are excluded from the oval-shaped VMH. The authors could clarify this, as in Extended Figure 6a.
- Figure 4a: There appear to be intensity differences in individual spots between the two panels. In principle, single-molecule methods should not produce differences in spot intensity regardless of expression levels. The method description needs to be more detailed. "To quantify Esr1 and OXTR expression at the single-cell level, the number of puncta in individual cells was counted using ImageJ." How many cells per animal were quantified? Were the cells counted manually or automatically? Was the counting performed blind?
- Figure 4: Both OXTR antagonist treatment and knockout reduced maternal aggression but did not completely eliminate it. The authors may wish to discuss this observation further—could other mechanisms be at play?
- Discussion on differences between male and maternal aggression: The authors compare mechanisms of aggression and

cite “repeated winning” as a factor that elevates VMHvl cell excitability and PA → VMHvl connectivity in males. However, this may not be a fair comparison, as mothers do not have the opportunity to “win repeatedly”. This process could still occur in females placed in a social competition context and might not represent a true sex difference.

- A potential typo in line 276: “OXTR also expresses abundantly in the VMHvl, and similar to PA” should be “OXTR is abundantly expressed...”
- Figure 5: Does the stimulation of PVN Oxt cells increase oxytocin levels in the blood?

Appropriate use of statistics and treatment of uncertainties: Mostly appropriate, except for the improvement on the single molecule spot counting raised above

References: appropriate credit to previous work?: Yes

Referee #2

(Remarks to the Author)

The regulation of the temporal specificity of maternal aggression represents an important topic in behavioral neuroscience. This study highlights the critical role of PA-Esr1 neurons, which project to the female's aggression center—VMHvl-Npy2r neurons—in facilitating maternal aggression in female mice. The authors identified two key forms of plasticity: enhanced connectivity from PA-Esr1 neurons to VMHvl-Npy2r neurons in the diestrus virgin and lactating states, and increased excitability of VMHvl-Npy2r neurons in lactating dams. These findings explain why VMHvl-Npy2r neuron activity is selectively elevated in lactating dams. Notably, oxytocin signaling in PA-Esr1 neurons plays an essential role in maternal aggression, providing a potential mechanism for the temporal specificity of maternal aggression linked to the presence of young offspring during lactation. Despite the compelling story, the manuscript contains three major weaknesses, as outlined below, which must be addressed before it can be considered for publication in Nature.

Major points:

1. While using an F1 hybrid may expedite the process by eliminating the need for backcrossing transgenic animals, I am concerned about the stability of the RI assay as presented in this manuscript. For instance, the average attack duration (per 10-minute assay) at PPD3–5 in the control group shows considerable variability: approximately 20 seconds (Figs. 1d, 1h, 2d, 3d); 30–50 seconds (assuming each trial represents 1 minute in Fig. 1u); 70 seconds (Fig. 4l); 40 seconds (Figs. 4p, 5h); 20–40 seconds (Fig. 5d); and 60 seconds (Fig. 5h, k). Some of the reported manipulation effects fall within this range of inter-group variability. Additionally, there is significant individual variation within each group. First, the authors must justify their choice of the F1 hybrid and demonstrate the reliability of their RI assay. Second, to enhance reliability, it is recommended that key experiments undergo re-annotation of behaviors by experimenters blinded to the experimental conditions, as unconscious bias could influence the identification of aggression behaviors, which are sometimes challenging to distinguish from other behaviors such as intensive investigation.

2. The necessity of VMHvl-projecting PA-Esr1 neuron activity for maternal aggression is convincingly demonstrated by the loss-of-function data (Figs. 1e–i and ED Fig. 2). However, the role of these neurons appears to be largely permissive, with limited evidence supporting an instructive function. The sole gain-of-function data suggesting an instructive role involves ChR2-mediated activation, which increased attack duration in dams but not in virgins (Figs. 1j–u). Similarly, although the permissive role of oxytocin signaling is well-documented (Figs. 4j–q and 5i–k), the mechanism by which oxytocin contributes to maternal aggression remains unclear.

To address this, the authors could use the pup separation paradigm (Fig. 5) to determine whether activation of PA-Esr1 neurons—or more specifically, VMHvl-projecting PA-Esr1 neurons—can facilitate maternal aggression. Furthermore, examining how oxytocin signals influence the activity of VMHvl-projecting PA-Esr1 neurons in vivo during the RI test would significantly strengthen the linkage between oxytocin signaling, activity dynamics, and behavioral outcomes.

3. Given the limited impact of VMHvl-projecting PA-Esr1 neuron activity on aggression in virgins (Fig. 1), the predominant mechanism underlying the rise and fall of maternal aggression appears to involve the gating of VMHvl-Npy2r neurons in terms of their excitability (Figs. 2j–t). Similarly, although the authors did not investigate the effect of PVN-oxytocin neuron activation on aggression in virgins, it is unlikely to evoke aggression, as oxytocin neuron activation typically promotes prosocial behaviors (e.g., PMID 32544386). This suggests that the temporal specificity of aggression primarily depends on unidentified mechanisms regulating VMHvl-Npy2r excitability.

To strengthen the manuscript in this aspect, it would be ideal if the authors could identify a molecular mechanism that regulates VMHvl-Npy2r neuron excitability in a lactation-specific manner. If this proves challenging, the textual tone should be moderated to explicitly state that a substantial portion of the temporal specificity of maternal aggression remains an open question. The authors could also discuss potential mechanisms, such as those informed by scRNA-seq data.

Minor points:

1. While the specificity of effectors is provided in certain experiments (Figs. 1f and 3b; although avoiding SEM for $n = 2$ data is recommended, as noted in Line 216), the targeting efficiency is not reported. It is crucial to specify approximately what percentage of VMHvl-projecting PA-Esr1 neurons were successfully manipulated.
2. It is advisable to remove error bars from $n = 2$ data (estrus) in Figs. 2f and 2g.
3. Although slightly beyond the scope of this manuscript, the observed estrus cycle-dependent changes in connectivity (Fig. 2b–g) are striking and intriguing. I wish future studies hopefully explore the mechanisms underlying this remarkable plasticity and its potential functional roles.
4. The data in Fig. 2u–y largely reiterate findings from previous research (ref. 10). These could be moved to an ED Fig to streamline the main figures. Additionally, the data presented in Fig. 3j–u appear more closely related to the content of Fig. 2.
5. Please clarify whether the animals used in ED Fig. 3 are the same as those used in Figs. 1e–i.
6. The data in ED Fig. 6 are highly intriguing; however, it is necessary to histochemically validate that mCherry is indeed

targeted to Npy2r–Esr1+ cells unless this has already been confirmed in previous studies.

Referee #3

(Remarks to the Author)

This manuscript is a very interesting and elegant study that follows previous papers from this distinguished group. Here, they unravel how PA(Esr1) to VMHvl(Npy2r) as well as PVN(OXT) to PA connections in the maternal brain of lactating mice trigger maternal, but not general, aggression by combining knowledge (as significantly contributed by the Lin group) on brain circuits involved in male and female, i.e., maternal, aggression. This group has largely contributed to our understanding of aggression circuits, especially involving the VMH.

The authors use a huge range of elegant and highly targeted techniques, for which this group is well known for. The data is – in the combination – novel, and their presentation is of high quality; the chosen colors in the figures are a matter of personal taste. The study design and the statistical analyses are sound, I could not detect any flaws. The Discussion is clear and follows their own and other significant publications.

It was very elegant to include social interest and receptivity after viral manipulation. The fiber photometry experiment is another highlight as it shows how specific cells respond to aggression-provoking cues thereby leading to aggression.

I have a few comments, that could clarify some aspects or help to improve this work.

F1 generation of C57 x SW females showed reliable maternal aggression. As such, I always worry that the authors might have created a rather artificial mouse line where the circuits are different from WT mice. Thus, it would be convincing to give a final proof in WT mice.

Did the authors make sure to test F1 females across different litters? How many litters have been used for the different mouse lines?

The change in maternal aggression intensity over the postpartum period as shown here in mice is very interesting and confirming previous mouse studies. Interestingly, such a decline in maternal aggression is not unique to mice as it has been shown for lactating rats as well (Caughey et al. 2011), hence being a rather general mechanism. That's probably to save the mothers' energy resources as soon as the pups become gradually more mobile – and being less in direct touch with the mother thereby being less of a positive stimulus to her.

In the Results, the sex of the juvenile intruder should be mentioned. In the Methods, it is mentioned that male and female intruders have been used; did the authors detect any differences in the mothers' behaviors towards the intruders based on their sex? Furthermore, I am a bit concerned regarding the definition of "juvenile", which is starting at 15 days of age; at that stage the offspring are still considered as pups and are not weaned from their mothers. As mice are alloparental, it is even more surprising why lactating mouse mothers attack those pups. In fact, I am wondering if the manipulations also made the mothers attack their own offspring? If so, this would suggest a different purpose that defending the offspring but rather a pup-attack circuit.

As the described pathway is only relevant for maternal aggression and cannot be stimulated in virgin females, potential differences in the expression of the investigated systems of NPY and ESR, including their receptors, need to be studied. Especially given that oxytocin signaling, which is increased in lactation (as has been shown also in the present study for the PA(Esr1)), can influence both systems – and vice versa.

It is very elegant to extend the studies to the oxytocin system in both PA and VMHvl. The findings suggest that oxytocin receptor signaling is more relevant in the PA than the VMH, at least in the specific cell populations investigated.

Furthermore, the PVN as source of oxytocin on the PA is striking.

Please provide the number of mice used for the cannula implantation.

As the aggression tests were performed in the home cage, please provide more information, i.e., were those standard mouse cages (supplier, product number, dimensions); such information would help to get a better idea of the overall conditions of the test.

I might have missed this information, but at which time of day (or phase of the light dark cycle) have the experiments been performed? The only information I could find was "...we performed the RI tests at approximately the same time the day...".

Version 2:

Reviewer comments:

Referee #1

(Remarks to the Author)

Comments on revised manuscript by Yamaguchi, Yan et al.

The revised manuscript addressed all of my comments. Therefore, I find the manuscript appropriate for publication in Nature with minor corrections. I would like to congratulate the authors on this major achievement linking maternal aggression and neuropeptide signaling.

Minor points:

- The current manuscript has many typos for example:

o Line 55 "meidal"

o Throughout the manuscript e.g. but not limited to line 192 to line 212: diestrous -> diestrus

o estrous: depends on the context - if meant as the specific phase of the estrous cycle, use estrus

o Figure 4a, 5s -> Oxt (as in mRNA, not protein)

- Line 81 to 106 – I found the beginning part of the manuscript could be in the methods, to focus on the main conclusions rather than on technical points.

Referee #2

(Remarks to the Author)

The authors have conducted a substantial number of additional experiments and data analyses that satisfactorily address all of my initial concerns. I now have no remaining issues. I appreciate their efforts and congratulate them on this significant milestone in behavioral neuroscience.

Referee #3

(Remarks to the Author)

The authors have revised their manuscript and replied to all my comments in a satisfactory manner. The work they put in is impressive. In my opinion, this study will make a highly valuable contribution how we see and understand maternal aggression.

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Responses to all reviewers

We thank all the reviewers for dedicating their valuable time to provide thoughtful and constructive feedback on our manuscript. Over the past nine months, we have added new experiments, performed additional analyses, and refined the introduction and discussion to address all reviewer comments. Here is a list of new experiments and analysis performed during the revision:

Behaviors:

- (1) Compared maternal aggression levels in C57, SW, and C57×SW F1 hybrid females across pre- and post-partum days (**Extended Data Fig. 1**).
- (2) Systematically quantified maternal aggression toward different types of intruders, including adult males, adult females, juvenile males, and juvenile females (**Extended Data Fig. 2**).
- (3) Tested the effect of adult male urine on maternal aggression (**Reviewer Fig. 4**).
- (4) Examined maternal aggression toward youngsters of different ages (**Reviewer Fig. 17**).

Histology:

- (1) Used CTB tracing to quantify the proportion of PA^{Esr1} cells projecting to the VMHvl and to evaluate HSV labeling efficiency (**Extended Data Fig. 3**).
- (2) Confirmed selective targeting of VMHvl^{Npy2r-Esr1+} cells using the CoffFon strategy (**Extended Data Fig. 9c, d**).
- (3) Quantified VMHvl Npy2r expression across estrous and lactation (**Extended Data Fig. 12b**).

Functional manipulations:

- (1) Tested the effect of OXTR antagonism in the PA on maternal aggression in both SW and C57 females (**Extended Data Fig. 11; Reviewer Fig. 14**).
- (2) Examined the effect of PA^{Esr1→VMHvl} terminal stimulation on aggression in lactating females following 24-hour pup separation (**Extended Data Fig. 13**).
- (3) Demonstrated increased serum oxytocin levels after 1-hour optogenetic activation of PVN^{OXt} cells (**Fig. 5g**).
- (4) Replicated the suppressive effect of chemogenetic PA^{Esr1→VMHvl} inhibition on maternal aggression in an independent cohort (**Reviewer Fig. 5**).

In vivo recordings:

- (1) Showed that PA^{Esr1→VMHvl} cell responses to pups decrease after pup separation and recover after 1-hour PVN^{OXt} activation (**Fig. 5m–r**).

Additional analyses:

- (1) Compared OXTR mRNA puncta intensity in the PA between virgin and lactating females (**Reviewer Fig. 2**).
- (2) Blindly re-annotated a subset of behavioral videos to confirm consistency and exclude unconscious bias (**Reviewer Fig. 7**).
- (3) Quantified intra- and inter-litter variability in maternal aggression (**Reviewer Fig. 15**).
- (4) Summarize the litter information for all experiments (**Reviewer Table 1**).

We also initiated new experiments to assess the contribution of Esr1-mediated signaling to VMHvl^{Npy2r} cell physiological property changes during lactation. These preliminary results are included in the reviewer figures.

Taken together, these additions substantially strengthen the manuscript. We are grateful for the reviewers' constructive feedback, which has greatly helped us improve the study.

Referee #1:

In this work, Yamaguchi et al. identify a two-tier neural mechanism that switches maternal aggression on and off. While it has long been known that mothers exhibit aggression towards intruders, the circuit basis for how this aggression is regulated remained unclear. Yamaguchi and colleagues uncover a neural pathway (PA → VMHvl) sufficient to trigger maternal aggression and identify synaptic and biophysical changes in PA and VMHvl that correlate with aggressive states. Moreover, they found that PA neurons projecting to VMHvl do not exhibit altered excitability in lactating mothers, suggesting an additional mechanism that enhances PA neuron output. They discovered that oxytocin signaling plays a crucial role in this process. Therefore, the rise of maternal aggression involves two mechanisms: first, biophysical changes in VMHvl neurons and changes in PA → VMHvl connectivity, and second, increased sensitivity to oxytocin signaling in PA neurons.

The major strengths of this work are threefold. First, the authors not only identify the critical roles of PA_Esr1 and VMHvl_Npy2r neurons in maternal aggression but also demonstrate that the monosynaptic connection between these neurons mediates this aggression. Importantly, they show that this pathway is specific to aggression and not involved in mating. Second, they combine in vivo and ex vivo analyses to pinpoint precise synaptic and biophysical changes across different estrous states and in lactating mothers. Third, through gain- and loss-of-function experiments, they demonstrate the key role of oxytocin in controlling this behavior.

Overall, this work represents a major milestone in understanding the circuit basis of maternal aggression. Several points could enhance the study's impact.

We thank the reviewer for their positive feedback and constructive comments.

First, the manuscript appears to be written primarily for specialists in the field of mammalian social behaviors. This is a missed opportunity. To reach a wider audience, the authors could include a more general introduction and Discussion, for example, on sexually dimorphic regulation of behavior and neuropeptide modulation of behaviors.

We thank the reviewer for this suggestion. We have now revised the introduction to highlight the gaps in the field and emphasize the broad questions under investigation as follows (**Lines 36-53**):

After birth, the young are vulnerable and helpless. To ensure their survival, mothers of many mammalian species vigorously attack intruders to protect their offspring from potential threats—a behavior known as maternal aggression (John and Corning 1973, Svare 1981, Lonstein and Gammie 2002). As its name suggests, maternal aggression is largely restricted to the maternal period. In mice and rats, non-lactating females rarely attack conspecific intruders; however, aggression increases sharply one day after parturition, peaks during the first postpartum week, then gradually declines and essentially disappears by the end of lactation (Svare and Gandelman 1973, Caughey, Klampfl et al. 2011). This striking behavioral transition is thought to arise from changes in the underlying aggression circuitry, and—given its autonomous nature—pregnancy- and lactation-related hormones have long been considered key contributors (Svare and Mann 1983).

In the late 1970s, seminal work by B. Svare and colleagues demonstrated that estrogen and progesterone regimens mimicking pregnancy, together with nipple development and pup suckling, were sufficient to induce aggression in virgin females, whereas sex hormones alone were ineffective (Svare and Gandelman 1976, Svare and Gandelman 1976, Svare and Mann 1983). Moreover, preventing pup suckling through thelectomy (nipple removal) blocked the emergence of maternal aggression in mouse mothers (Svare and Gandelman 1976). These findings indicate that hormonal changes during pregnancy, combined with postpartum nursing, are essential for initiating maternal aggression. Despite these important insights, how the female aggression circuit is reorganized during pregnancy and how pup suckling promotes maternal aggression remain unclear nearly five decades later.

We additionally added a paragraph to discuss the potential role of oxytocin in male aggression in our Discussion as follows (**Lines 470-479**):

*OXTR is also abundantly expressed in the male PA. Could oxytocin similarly modulate intruder-directed aggression in fathers? This seems plausible at least in biparental species such as California mice, in which fathers exhibit heightened “paternal aggression” paralleling maternal aggression (Trainor, Finy et al. 2008). Although males do not nurse, oxytocin can still be released during physical interactions with infants (Morris, Turner et al. 2021). Supporting this idea, a recent mouse study reported strengthened excitatory input from the lateral hypothalamus to PVN^{OXT} neurons in fathers compared to virgin males (Inada, Hagihara et al. 2022), providing a potential mechanism for increased oxytocin release during fatherhood. Future studies should determine whether male house mice (*Mus musculus*) also exhibit enhanced aggression during parenthood and whether PA oxytocin signaling contributes to “paternal aggression” or other oxytocin-linked agonistic behaviors.*

Second, while the authors convincingly show that the neural basis of aggression differs between lactating females and males, they have not demonstrated the exact link between sex differences and the observed phenomena. For example, what is the molecular basis of Oxt induction in mothers?

Several lines of evidence suggest that estrogen, acting through *Esr1*, regulates OXTR expression. First, OXTR expression is markedly reduced in ovariectomized female mice, and this reduction is rescued by exogenous E2 (Sharma, LeBlanc et al. 2019). Second, although estrogen robustly increases OXTR binding in multiple brain regions, including the PA, in wild-type females, it fails to do so in *Esr1*-knockout mice (Young, Wang et al. 1998). Third, the mouse OXTR promoter contains both half and full estrogen response elements (EREs), enabling *Esr1*-dependent transcriptional regulation (Mamrut, Harony et al. 2013). Thus, the OXTR induction in mothers is likely caused by estrogen surge during pregnancy, as suggested previously (Meddle, Bishop et al. 2007, Insel 2008).

We have now added a sentence in the Discussion to include this information (**Lines 455-457**):

*During lactation, OXTR expression markedly increases in the VMHvl, PA, and many other limbic regions, likely driven by *Esr1* activation following the pregnancy-associated estrogen surge (Young, Wang et al. 1998, Insel 2008).*

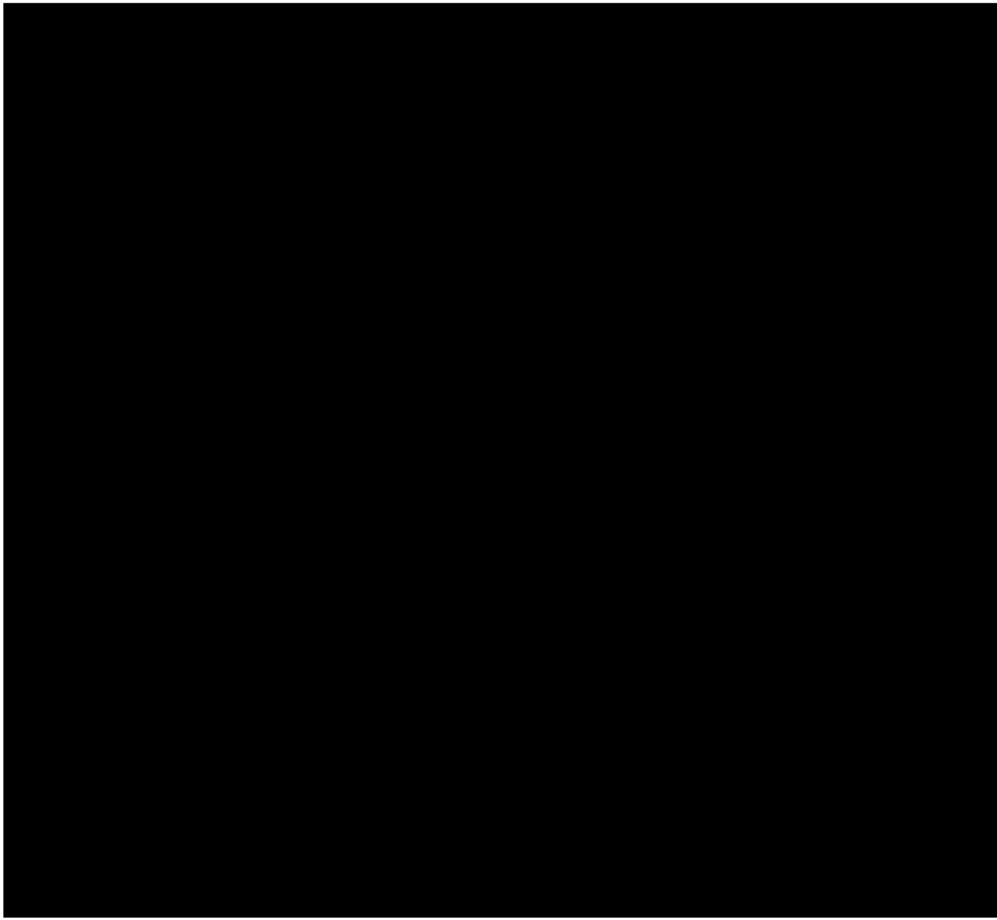
The authors suggest that “The synaptic and cellular changes, likely induced by the cocktail of sex hormone surges during pregnancy and parturition, primed the aggression circuit to be ready to engage,” but directly testing this hypothesis would significantly enhance the study’s impact.

We agree with the reviewer that understanding how sex hormones remodel the aggression circuit during pregnancy and parturition is an important and timely question. However, this is a complex problem that cannot be fully resolved with one or two additional experiments. Indeed, Dr. Kohl’s group recently published an elegant study devoted entirely to dissecting how estrogen and progesterone alter MPOA galanin neuron properties during pregnancy (Ammari, Monaca et al. 2023), illustrating the scope and depth required to address such questions thoroughly.

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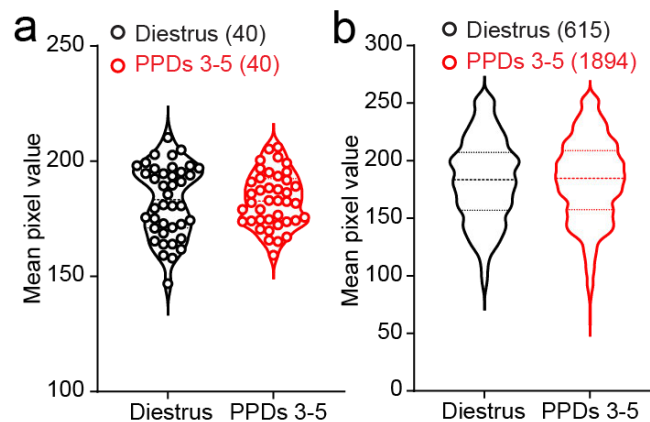
Third, several minor points could be clarified:

- Figure 1j: Please indicate where VMHvl is in this panel. It appears that most of the green signals are excluded from the oval-shaped VMH. The authors could clarify this, as in Extended Figure 6a.

We have now marked the VMHvl boundary in all histological images. In the **original Figure 1j** (now Figure 1f), ChR2–EYFP is primarily expressed within the VMHvl. Because ChR2–EYFP is membrane-bound, it is also present in VMHvl^{Npy2r} axons that extend beyond the VMHvl boundary. The ChR2–EYFP signals in the original **Extended Data Figure 6a** (now Extended Data Figure 9a) arise from PA^{Esr1} axons, which are distributed both within the VMHvl and in the surrounding VMHvl “shell.” VMHvl neurons are known to extend dendrites into this shell region to form synapses with axons originating from distal areas (Eugene Millhouse 1973).

- Figure 4a: There appear to be intensity differences in individual spots between the two panels. In principle, single-molecule methods should not produce differences in spot intensity regardless of expression levels. The method description needs to be more detailed. “To quantify Esr1 and OXTR expression at the single-cell level, the number of puncta in individual cells was counted using ImageJ.” How many cells per animal were quantified? Were the cells counted manually or automatically? Was the counting performed blind?

The cells were counted manually and in a blinded manner. For each image, we randomly selected 10 cells and quantified the number of puncta using ImageJ. We have now also analyzed puncta intensity by calculating the mean pixel value for each punctum and found no difference in average puncta intensity between PA^{OXTR} cells in virgin and lactating females (**Reviewer Figure 2**). The puncta appear brighter in lactating females likely because they are more numerous, not because each punctum is more intense.



Reviewer Figure 2 Distribution of OXTR punctum intensity of PA^{OXTR} cells between virgin and lactating females.

a. No difference in the average punctum intensity per PA^{OXTR} cell between virgin vs. lactating females. N = 40 cells from 4 animals.

b. No difference in punctum intensity in PA^{OXTR} cells from virgin vs. lactating females. N = 615 (diestrus) and 1894 (PPDs 3-5) puncta from 4 animals.

Dashed lines indicate median, 25% and 75%. (a) Unpaired t-test and (b) Mann-Whitney test.

- Figure 4: Both OXTR antagonist treatment and knockout reduced maternal aggression but did not completely eliminate it. The authors may wish to discuss this observation further—could other mechanisms be at play?

The reviewer correctly pointed out that maternal aggression was reduced but not abolished after OXTR antagonist treatment or OXTR knockout. This outcome is consistent with the decreased, but not fully eliminated, maternal aggression observed 24 hours after pup separation. We believe the partial reduction reflects the mechanism by which OXTR signaling modulates PA output. Rather than acting as a gate, OXTR activation amplifies PA cell excitability and enhances PA-driven VMHvl output. Thus, in the absence of oxytocin, PA cells can still activate VMHvl neurons, but with reduced efficiency, leading to attenuated rather than abolished maternal aggression.

From a technical perspective, incomplete knockout efficiency may also contribute to the residual behavior. However, the OXTR antagonist should block all OXTR in PA cells, yet its effect is weaker than that of genetic knockout. This likely reflects the slow dynamics of OXTR signaling: OXTR activation induces cellular changes that may persist after acute antagonist application. In contrast, the oxytocin effect on PA cells should be completely absent after OXTR knockout. Overall, the reduced, but not eliminated, maternal aggression after OXTR manipulations is consistent with oxytocin's slow action and its role in amplifying, rather than gating, PA output.

We have now added two sentences in our Discussion to explain the reduced but not eliminated maternal aggression after OXTR manipulation:

In the PA, OXTR is broadly and strongly expressed in nearly all PA^{Esr1} cells, allowing oxytocin to increase their input–output gain by elevating input resistance, potentially through the closure of specific potassium channels (Liu, Eyring et al. 2022). (Newly added, lines 460-462) Because this effect builds slowly, it may explain the gradual decline in maternal aggression after pup separation and the moderate reduction observed following acute OXTR antagonism in the PA.

Notably, after OXTR knockout in the PA, maternal aggression is heavily suppressed but not eliminated. The remaining aggression likely reflects an amplifying rather than an absolute gating role of oxytocin: even in the absence of oxytocin, PA input can still drive VMHvl activity to some degree. (Lines 466-469)

- Discussion on differences between male and maternal aggression: The authors compare mechanisms of aggression and cite “repeated winning” as a factor that elevates VMHvl cell excitability and PA → VMHvl connectivity in males. However, this may not be a fair comparison, as mothers do not have the opportunity to “win repeatedly”. This process could still occur in females placed in a social competition context and might not represent a true sex difference.

This is a good point. Based on the reviewer's comments, we have now revised the Discussion as follows (Lines 428-439):

Connection strength also differs markedly between sexes. In males, PA-VMHvl input onto individual VMHvl^{Esr1} cells can readily increase: in 5-day and 10-day winners, PA-evoked EPSC amplitudes are roughly twice those of naïve males (Yan, Wei et al. 2024). By contrast, PA-evoked

EPSC amplitudes onto VMHvl^{Npy2r} cells do not differ between diestrous virgins and lactating females, and both resemble the oEPSC amplitude observed in naïve males. Thus, in males, PA input onto individual VMHvl cells is consistently present and further potentiated by winning, whereas in females, PA-VMHvl connectivity varies with reproductive state and, when present, generally remains weak.

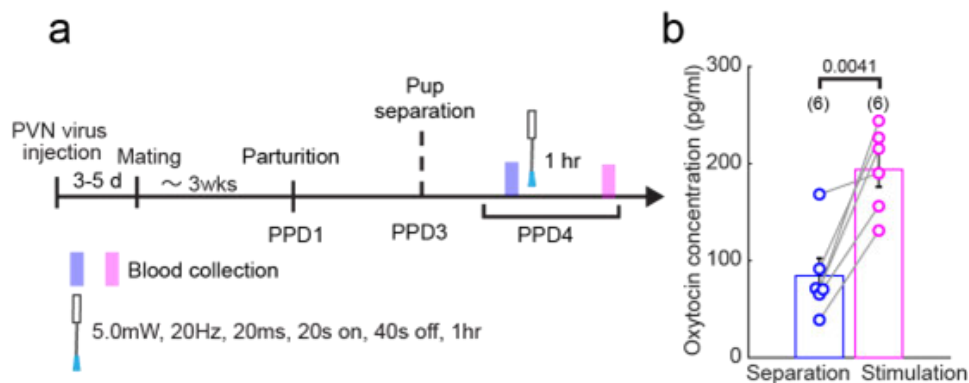
We did not evaluate potential winning-induced PA-VMHvl potentiation in females, as virgin females rarely show robust aggression, and repeated winning in either virgin or lactating females does not reliably increase aggression (Hood 1988). Nonetheless, it remains possible that PA-VMHvl connections could be further modified by social experience in the subset of females that do exhibit winning-induced aggression escalation.

- A potential typo in line 276: “OXTR also expresses abundantly in the VMHvl, and similar to PA” should be “OXTR is abundantly expressed...”

We have now changed the text accordingly (**Line 309**).

- Figure 5: Does the stimulation of PVN Oxt cells increase oxytocin levels in the blood?

We have now measured the serum oxytocin level before and after 1-hour PVN OXT cell stimulation and found a significant elevation of oxytocin concentration as expected (**Reviewer Figure 3**). The data is now presented in **Figure 5g**.



Reviewer Figure 3: Increased serum oxytocin level after PVN^{OXT} cell activation.

- Experimental schematics to examine the effect of PVN^{OXT} stimulation on circulating oxytocin level.
- Serum oxytocin level in PPD4 females 24 hours after pup separation before (blue) and after (magenta) light activation to PVN^{OXT} cells. Numbers in parentheses indicate the number of animals. Statistical significance was determined using (b) paired t-test.

Referee #2

The regulation of the temporal specificity of maternal aggression represents an important topic in behavioral neuroscience. This study highlights the critical role of PA-Esr1 neurons, which project to the female's aggression center—VMHvl-Npy2r neurons—in facilitating maternal aggression in female mice. The authors identified two key forms of plasticity: enhanced connectivity from PA-Esr1 neurons to VMHvl-Npy2r neurons in the diestrus virgin and lactating states, and increased excitability of VMHvl-Npy2r neurons in lactating dams. These findings explain why VMHvl-Npy2r neuron activity is selectively elevated in lactating dams. Notably, oxytocin signaling in PA-Esr1 neurons plays an essential role in maternal aggression, providing a potential mechanism for the temporal specificity of maternal aggression linked to the presence of young offspring during lactation. Despite the compelling story, the manuscript contains three major weaknesses, as outlined below, which must be addressed before it can be considered for publication in Nature.

We thank the reviewer for their positive, constructive, and specific feedback. We have addressed all of the reviewer's comments with new experiments and corresponding revisions to the manuscript.

Major points:

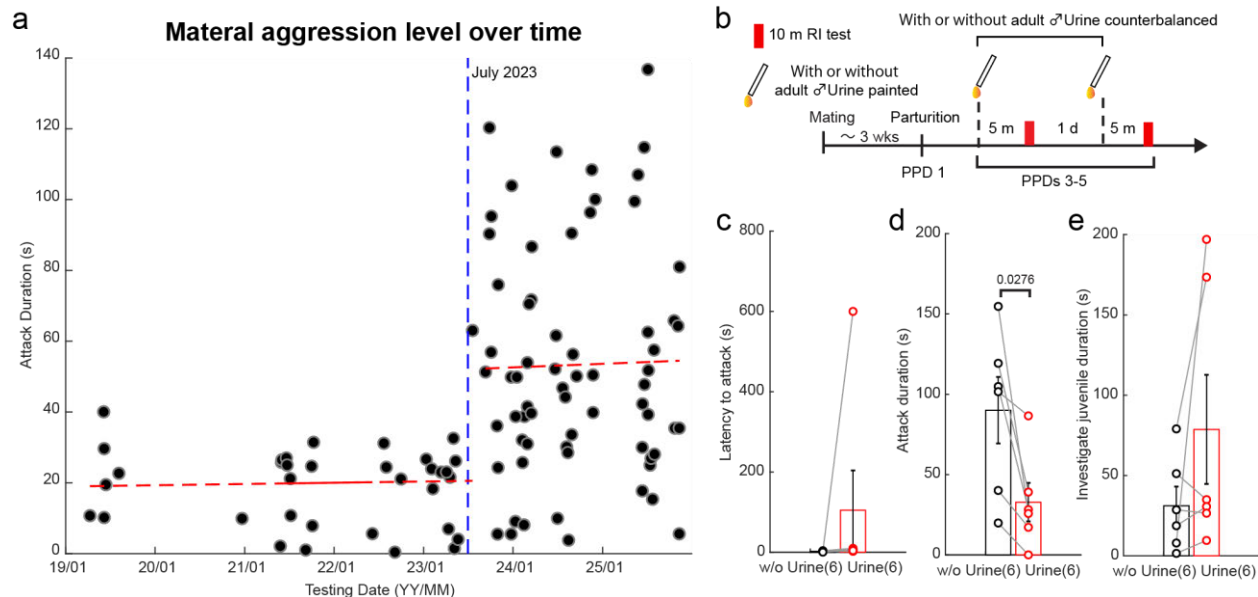
While using an F1 hybrid may expedite the process by eliminating the need for backcrossing transgenic animals, I am concerned about the stability of the RI assay as presented in this manuscript. For instance, the average attack duration (per 10-minute assay) at PPD3–5 in the control group shows considerable variability: approximately 20 seconds (Figs. 1d, 1h, 2d, 3d); 30–50 seconds (assuming each trial represents 1 minute in Fig. 1u); 70 seconds (Fig. 4l); 40 seconds (Figs. 4p, 5h); 20–40 seconds (Fig. 5d); and 60 seconds (Fig. 5h, k). Some of the reported manipulation effects fall within this range of inter-group variability. Additionally, there is significant individual variation within each group.

We thank the reviewer for pointing out the variability in baseline maternal aggression across our datasets. In response, we conducted an extensive investigation to identify potential contributing factors. We assessed the age of the test mice, transgenic line identity, litter effects, parental age, breeding schemes, and housing facility (we maintain three mouse facilities), but none of these variables explained the observed variability.

Eventually, we noticed an abrupt increase in baseline maternal aggression around July 2023. Before July 2023, the average attack duration was approximately 20 s, whereas after July 2023 this average increased to ~50 s (**Reviewer Fig. 4a**). This shift coincided with a change in experimental location—from a shared behavioral rig to a dedicated rig. The shared rig housed other ongoing projects involving adult males and occasional predator odors, whereas the dedicated rig was used exclusively for the maternal aggression project.

To directly test whether environmental cues influence baseline maternal aggression, we applied adult male urine to juvenile intruders and observed a reduction in maternal aggression (**Reviewer Fig. 4b–e**), consistent with our finding that maternal females show lower aggression toward adult

males compared to juveniles (see **Extended Data Fig. 2**). These results strongly support the idea that the environmental context contributed to the shift in baseline aggression levels.



Reviewer Figure 4: Adult male cues suppress maternal aggression toward juveniles.

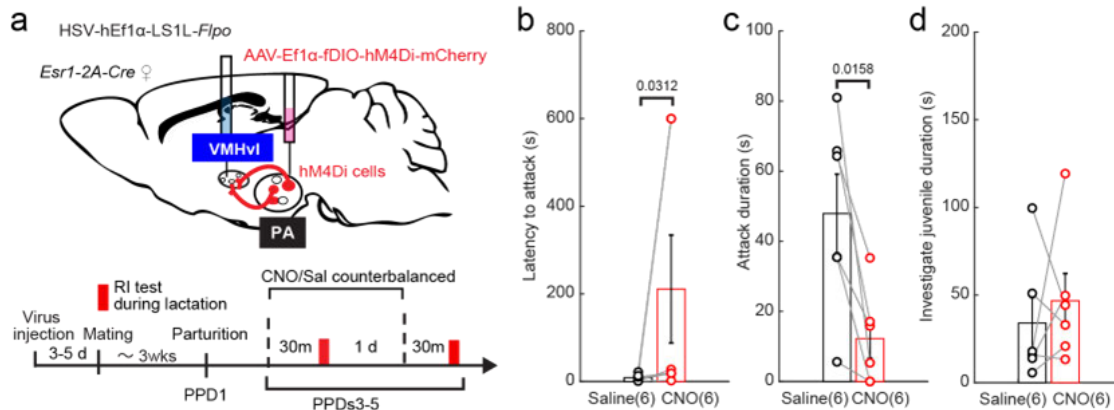
a. Experimental schematics and test schedule.

b–d. Latency to attack (**b**), attack duration (**c**), and investigation duration (**d**) toward a juvenile intruder presented with or without painted adult male mouse urine.

Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of subject animals. Statistical significance was assessed using the Wilcoxon matched-pairs signed-rank test for (**b**) and paired t-tests for (**c**, **d**). All p values < 0.05 are indicated.

Importantly, for every experiment in our study, test and control animals were always evaluated in parallel. With the exception of the PA and VMHvl OXTR knockout experiments (**Fig. 4n–q**; **Extended Data Figs. 12i–l**), all experiments were designed to compare aggression both between and within animals (e.g., OXTR antagonist vs. saline, saline vs. CNO, light-on vs. light-off). For the OXTR knockout experiments, GFP and Cre groups were tested side-by-side. Therefore, we are confident that the results of each experiment and the overall conclusions of the study is valid despite the baseline shift.

As an additional validation, we repeated the PA^{Esr1→VMHvl} chemogenetic inhibition experiment in a new cohort of females that exhibited a higher baseline aggression level (average attack duration after saline injection: 47.94 ± 11.23 s). Consistent with the original results (**Fig. 1a–e**), chemogenetic inhibition significantly reduced maternal aggression (**Reviewer Fig. 5**).



Reviewer Figure 5: Inhibition of PA^{Esr1}-VMHvl pathway reliably suppresses maternal aggression.

a, Experimental schematics and test schedule.

b, c, In the hM4Di group, CNO injection increased the latency to attack (**b**) and decreased attack duration (**c**). For animals that did not attack, latency was assigned a value of 600 s.

d, Juvenile investigation duration did not differ between CNO and saline conditions.

Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of subject animals. Statistical significance was assessed using the Wilcoxon matched-pairs signed-rank test for (**b**) and paired t-tests for (**c**). All *p* values < 0.05 are indicated.

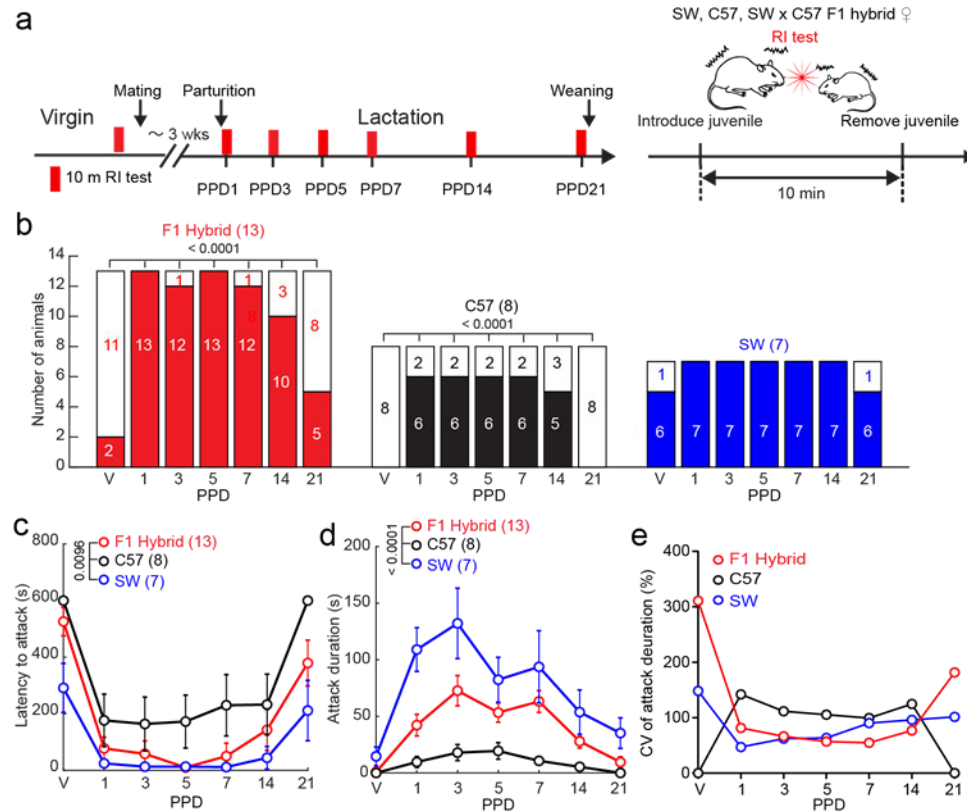
First, the authors must justify their choice of the F1 hybrid and demonstrate the reliability of their RI assay.

We chose to use the C57 $\times$ SW F1 hybrid because these mice are genetically identical: for every gene, each animal receives one allele from C57 and one from SW. Therefore, they are not more genetically variable than their parent strains. This hybrid strategy is well established and has been used in previous studies to generate genetically uniform transgenic mice with improved behavioral robustness, including aggression, relative to C57 mice (Golden, Jin et al. 2019, Sloin, Bikovski et al. 2022).

Since summer 2023, the average maternal aggression level of our F1 hybrids has remained stable (**Reviewer Fig. 4a**). However, we consistently observe substantial variability across individuals, with total attack duration ranging from 3.8 to 136.7 s in the 10-min resident-intruder test. To determine whether hybrid females are more variable than SW or C57 females, we directly compared maternal aggression across postpartum days in SW, C57, and C57 $\times$ SW females using the same designated behavioral rig (**Reviewer Fig. 6**). Although the three strains differ in average aggression levels (SW > C57 $\times$ SW > C57), the coefficient of variation (CV) of attack duration of the F1 hybrids is comparable to that of SW mice and lower than that of C57 mice.

The large variability in maternal aggression among individuals with identical genetic backgrounds is not unexpected. In males, we and others have repeatedly reported that genetically identical C57 animals can range from completely non-aggressive to highly aggressive (Falkner, Grosenick et al. 2016, Stagkourakis, Spigolon et al. 2018, Stagkourakis, Spigolon et al. 2020, Yan, Wei et

al. 2024, Dai, Zheng et al. 2025). Taken together, our data indicate that C57 × SW F1 hybrid mothers are at least as reliable as SW mothers, and more reliable than C57 mothers, in the resident–intruder assay, despite individual variability. These new behavioral results have been incorporated into **Extended Data Figure 1**.



Reviewer Figure 6. Comparison of maternal aggression among C57 × SW F1 hybrids, C57, and SW mice.

a, Experimental timeline.

b, Number of females from different strains (C57 × SW F1 hybrids, C57BL/6N, and SW) that attacked a juvenile intruder during the 10-min RI tests across reproductive states. V, virgin.

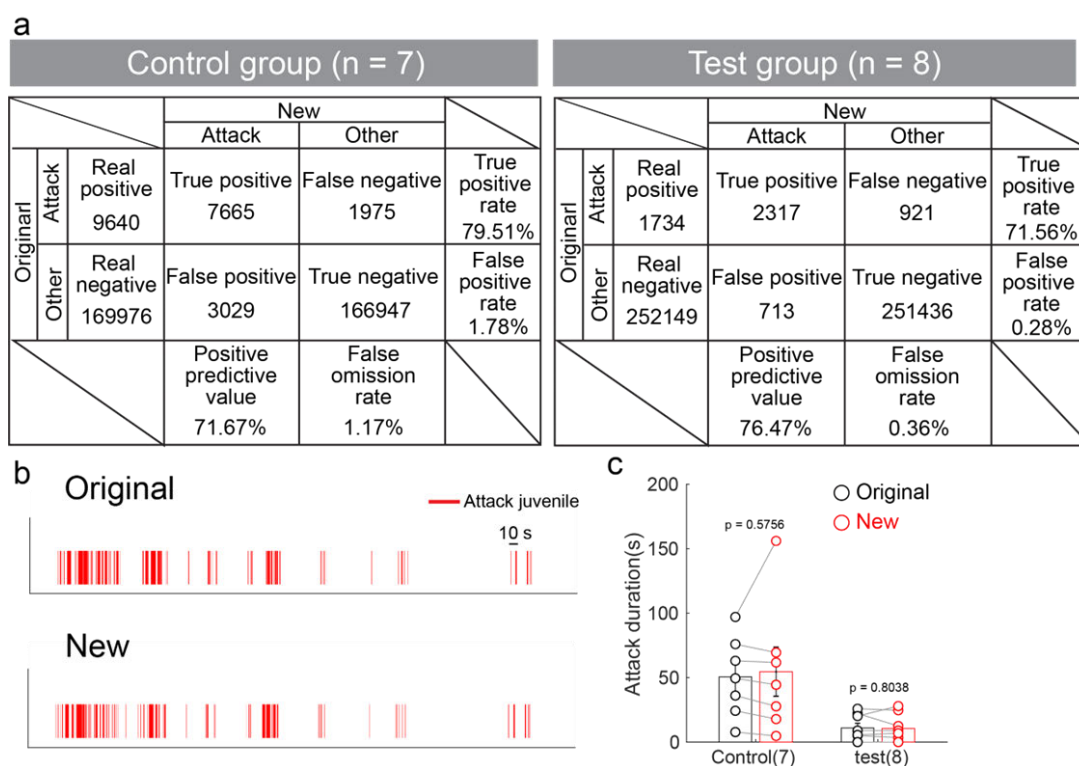
c, **d**, Attack latency (**c**) and duration (**d**) against a juvenile intruder in females from different strains (C57 × SW F1 hybrids, C57BL/6N, and SW). V, virgin.

d, The coefficient of variation (CV) of attack duration across days in females from different strains. V, virgin.

Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of animals. Statistical significance was determined using (**b**) Chi-square test, and (**c**, **d**) mixed-effect model with repeated measures followed by Bonferroni's multiple-comparison test. Post-hoc test results are not shown. See Supplementary Table 1, EDF 1k, 1l for statistical details.

Second, to enhance reliability, it is recommended that key experiments undergo re-annotation of behaviors by experimenters blinded to the experimental conditions, as unconscious bias could influence the identification of aggression behaviors, which are sometimes challenging to distinguish from other behaviors such as intensive investigation.

Attack is a relatively obvious behavior with distinct motor patterns. The consistency of human-to-human annotation of male attack is approximately 0.8 (frame-level F1 score) (Burgos-Artizzu, Dollár et al. , Segalin, Williams et al. 2021). To assess potential unconscious bias in our behavioral annotation, a blinded experimenter re-annotated all videos from the OXTR conditional knockout experiment. The frame-level F1 scores between the original and blinded annotations were 0.753 and 0.739 for the control and test groups, respectively. These results indicate no detectable bias favoring the expected outcomes in the original annotation (**Reviewer Figure 7**). Therefore, we retained the original annotations for the remainder of the analyses.



Reviewer Figure 7 Comparison of the original annotation and new annotation.

a, Confusion matrix to compare attack annotations between original and new files in both the test and control groups. The new annotations were performed blind to group identity.

b, Raster plots comparing the annotations of one sample video by two different annotators.

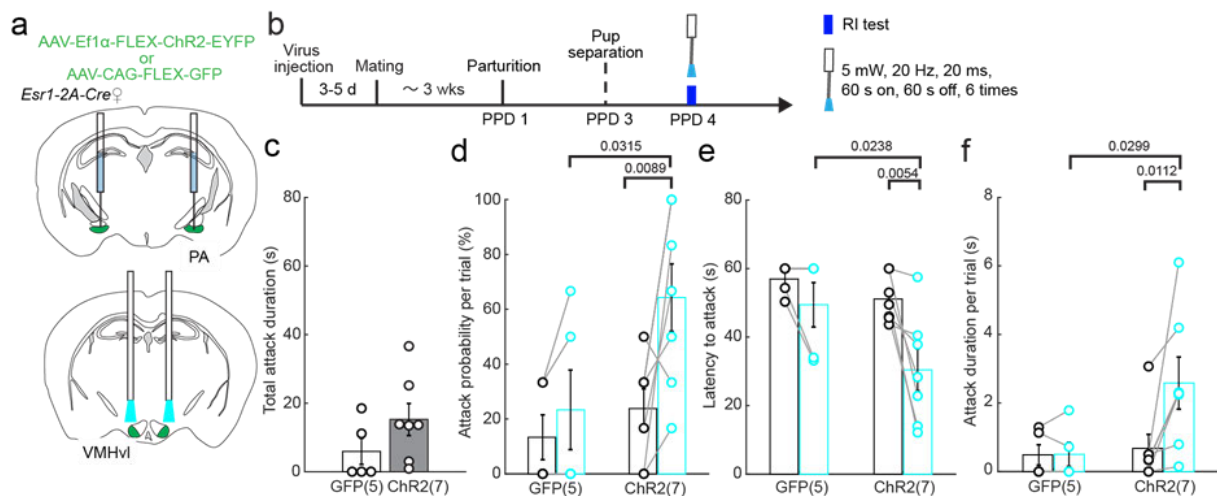
c, Comparison of attack duration between the original and new annotation files in both the test and control groups. Statistical significance was assessed by paired t-test. Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of animals.

The necessity of VMHvl-projecting PA-Esr1 neuron activity for maternal aggression is convincingly demonstrated by the loss-of-function data (Figs. 1e–i and ED Fig. 2). However, the

role of these neurons appears to be largely permissive, with limited evidence supporting an instructive function. The sole gain-of-function data suggesting an instructive role involves ChR2-mediated activation, which increased attack duration in dams but not in virgins (Figs. 1j–u). Similarly, although the permissive role of oxytocin signaling is well-documented (Figs. 4j–q and 5i–k), the mechanism by which oxytocin contributes to maternal aggression remains unclear.

To address this, the authors could use the pup separation paradigm (Fig. 5) to determine whether activation of PA-Esr1 neurons—or more specifically, VMHvl-projecting PA-Esr1 neurons—can facilitate maternal aggression.

As suggested by the reviewer, we examined the effect of PA^{Esr1}-VMHvl projection in promoting maternal aggression after pup separation. Our data shows that optogenetic stimulation of the pathway in lactating females with separated pups is sufficient to evoke time-locked attack (**Reviewer Figure 8**). This result is consistent with the idea that the lack of maternal aggression after pup separation is due to decreased output of PA^{Esr1} cells, whereas the downstream circuit, e.g., synaptic connection from PA^{Esr1} to VMHvl and VMHvl^{Npy2r} cell excitability, remains effective in driving the behavior. Thus, when we artificially enhance the output of PA^{Esr1} cells to the VMHvl, we can induce attack. This new result is now presented in **Extended Data Figure 13**.



Reviewer Figure 8. Optogenetic activation of PA^{Esr1}→VMHvl cells is effective in promoting attack in lactating females following 24-hour pup separation.

a, Experimental schematics.

b, Experimental timeline used to examine the effect of PA^{Esr1}→VMHvl stimulation on maternal aggression towards juvenile intruders following pup separation.

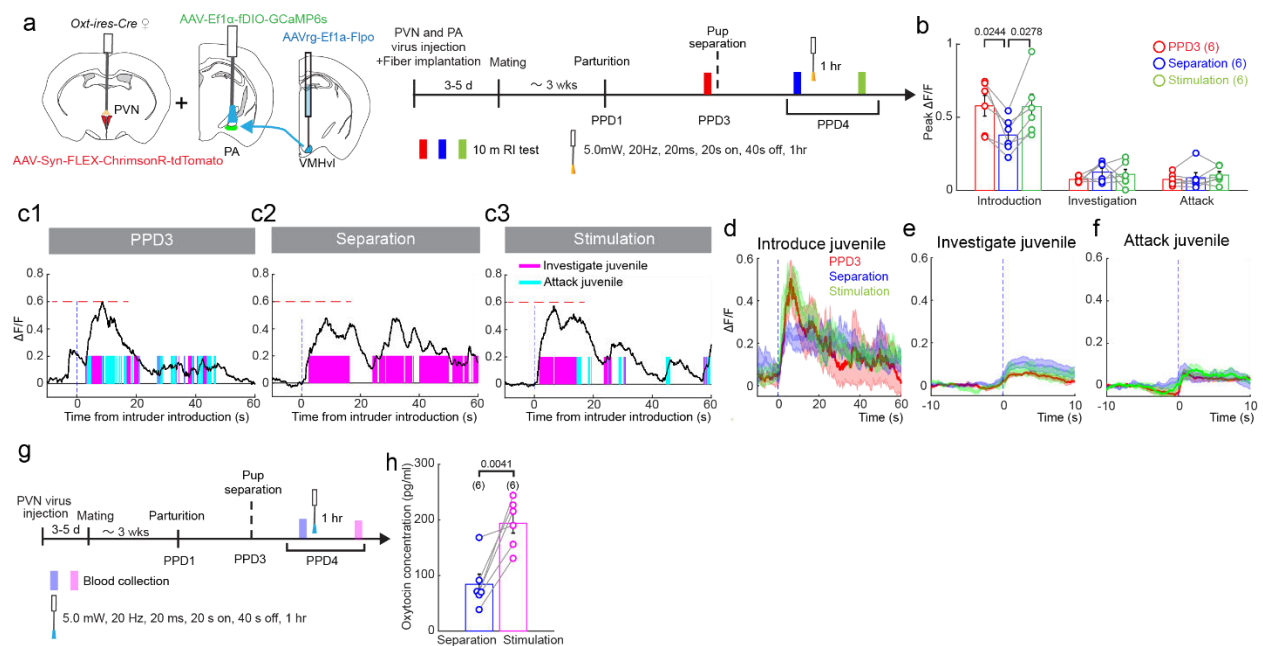
c, Total attack duration of ChR2 (n = 7) and GFP (n = 5) lactating females. Mann-Whitney test.

d-f, Following 24-hour pup separation, light stimulation in lactating ChR2 animals, but not GFP animals, (**d**) increased attack probability, (**e**) decreased attack latency, (**f**) and increased the average attack duration per trial. N = 5 (GFP) and 7 (ChR2). Mixed effect model with repeated measure followed by Bonferroni's multiple-comparison test.

Bars and error bars represent mean ± s.e.m. . All p values < 0.05 are indicated.

Furthermore, examining how oxytocin signals influence the activity of VMHvl-projecting PA-Esr1 neurons in vivo during the RI test would significantly strengthen the linkage between oxytocin signaling, activity dynamics, and behavioral outcomes.

We thank the reviewer for this excellent suggestion. We have now recorded the in vivo responses of VMHvl-projecting PA^{Esr1} neurons before and after pup separation, and after optogenetic stimulation of PVN^{OXT} cells. We found that the response of VMHvl-projecting PA^{Esr1} cells to a juvenile intruder decreases after pup separation, and 1-hour PVN^{OXT} cell activation restores the response (Reviewer Figure 9a-f). Additionally, we confirmed that PVN^{OXT} activation significantly increases the serum OXT level (Reviewer Figure 9g, h). These results strengthen the conclusion that pup separation diminishes PA^{Esr1} cell output, likely due to decreased OXT level. These new results are now shown in Figure 5g and 5m-r.



Reviewer figure 9. PVN^{OXT} cell activation restores reduced VMHvl-projecting PA cell responses to juveniles due to pup separation.

a, Experimental schematics.

b, Peak Ca²⁺ signal of VMHvl-projecting PA cells during intruder introduction, investigation, and attack.

c1-3, Representative Ca²⁺ traces of VMHvl-projecting PA cells in a ChrimsonR-expressing animal during interaction with a juvenile intruder before pup separation (left), 24 hours after pup separation (center), and following 1-hour light stimulation of PVN^{OXT} cells (right). Color shading indicates behavioral episodes.

d-f, PETHs of Ca²⁺ signals from VMHvl-projecting PA cells aligned to intruder introduction (d), investigation (e), and attack (f) in PPD3 females (red), PPD4 females after pup separation (blue), and following PVN^{OXT} stimulation (green).

g, Experimental schematics to examine the effect of PVN^{OXT} stimulation on serum oxytocin level.

h, The serum oxytocin levels in PPD4 females 24 hours after pup separation before (blue) and after (magenta) light activation to PVN^{OX^T} cells.

Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of animals. Statistical significance was determined using **(b)** one-way ANOVA with repeated measures followed by Tukey's multiple-comparison test, and **(h)** unpaired t-test. All p values < 0.05 are indicated.

3. Given the limited impact of VMHvl-projecting PA-Esr1 neuron activity on aggression in virgins (Fig. 1), the predominant mechanism underlying the rise and fall of maternal aggression appears to involve the gating of VMHvl-Npy2r neurons in terms of their excitability (Figs. 2j–t). Similarly, although the authors did not investigate the effect of PVN-oxytocin neuron activation on aggression in virgins, it is unlikely to evoke aggression, as oxytocin neuron activation typically promotes prosocial behaviors (e.g., PMID 32544386). This suggests that the temporal specificity of aggression primarily depends on unidentified mechanisms regulating VMHvl-Npy2r excitability.

To strengthen the manuscript in this aspect, it would be ideal if the authors could identify a molecular mechanism that regulates VMHvl-Npy2r neuron excitability in a lactation-specific manner. If this proves challenging, the textual tone should be moderated to explicitly state that a substantial portion of the temporal specificity of maternal aggression remains an open question. The authors could also discuss potential mechanisms, such as those informed by scRNA-seq data.

We agree with the reviewer that elucidating the molecular mechanisms underlying the change of VMHvl^{Npy2r} cell excitability is an important next step. However, it is a non-trivial question that cannot be addressed with one or two experiments.

[REDACTED]



[REDACTED]

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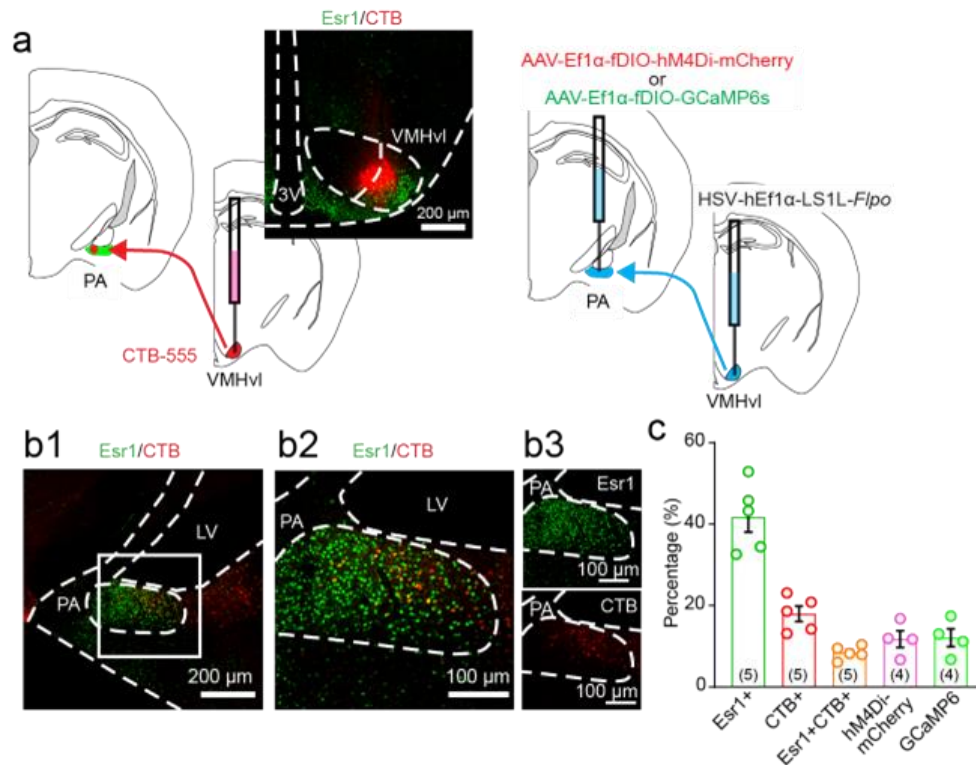
The reviewer is correct that the VMHvl^{Npyr2r} cell excitability change is an important contributor to the maternal aggression. We have added a paragraph to discuss the critical role of PA^{Esr1}-VMHvl circuit change for maternal aggression and the unidentified molecular mechanisms underlying these changes as follows (**Lines 412-419**):

After 24 hours of pup separation, natural maternal aggression was reduced; however, PA^{Esr1} to VMHvl terminal stimulation still effectively promoted attack. This suggests that both VMHvl^{Npyr2r} cell excitability and PA^{Esr1} to VMHvl synaptic strength likely remain high. Such sustained circuit properties would allow maternal aggression to rebound quickly once the mother regains access to her pups. We speculate that these PA^{Esr1}-VMHvl circuit modifications are induced by the hormonal milieu of pregnancy and are largely independent of oxytocin levels during lactation. The molecular mechanisms underlying the enhanced VMHvl excitability and strengthened PA^{Esr1} to VMHvl connectivity remain to be elucidated in future studies.

Minor points:

1. While the specificity of effectors is provided in certain experiments (Figs. 1f and 3b; although avoiding SEM for n = 2 data is recommended, as noted in Line 216), the targeting efficiency is not reported. It is crucial to specify approximately what percentage of VMHvl-projecting PA-Esr1 neurons were successfully manipulated.

In an effort to estimate the total number of VMHvl-projecting PA^{Esr1} cells, we injected a widely used retrograde tracer, cholera toxin b subunit (CTB), into the VMHvl and stained Esr1 in the PA (**Reviewer Figure 11a, b**). We found that approximately ~20% of total PA cells (identified by DAPI) project to the VMHvl, and ~50% of those cells co-express Esr1 (**Reviewer Figure 11c**). Thus, VMHvl-projecting PA^{Esr1} cells represent ~10% of total PA cells in female mice. These numbers are similar to what we previously reported in male mice (Yamaguchi, Wei et al. 2020). In our chemogenetic and fiber photometry experiments, the infected cells, i.e., VMHvl-projecting PA^{Esr1} cells, also constitute ~10% of total PA cells (**Reviewer Figure 11b, c**), indicating that the HSV viral strategy captured the majority of the cells of interest. This data is now presented in **Extended Data Figure 3**. In our experience, different retrograde viruses vary in their labeling efficiency across brain regions. HSV appears to be particularly effective for labeling PA cells.



Reviewer Figure 11: Comparison of the retrograde labeling efficiency of HSV versus CTB.

a, (Left) Experimental schematics and representative images showing Esr1 expression (green) and Alexa Fluor 555 Conjugated CTB (red) injected into the VMHvl. Scale bar, 200 μ m. 3V, third ventricle. (Right) Schematics showing the viral strategy to label PA cells projecting to the VMHvl.

b, Representative images showing Esr1 expression (green) and CTB retrogradely labeled from the VMHvl (red) in the PA. Panels (**b2**) and (**b3**) show enlarged views of the boxed area in (**b1**). Scale bars: 200 μ m (**b1**) and 100 μ m (**b2**, **b3**).

c, Percentage of Esr1-positive, CTB-positive, CTB/Esr1 double-positive, hM4Di-mCherry-expressing, and GCaMP6f-expressing cells among all PA cells, based on DAPI staining. Note that the percentages of hM4Di-mCherry and GCaMP6f cells are comparable to CTB/Esr1 double-positive cells. Bars and error bars represent mean $\pm$ s.e.m., and numbers in parentheses indicate the number of animals. Statistical analysis was performed using ordinary one-way ANOVA followed by Tukey's multiple-comparison test among the Esr1⁺CTB⁺, hM4Di, and GCaMP6f groups. All p values < 0.05 are indicated.

2. It is advisable to remove error bars from n = 2 data (estrus) in Figs. 2f and 2g.

Thanks for the suggestion. We have removed the error bars in Figs. 2f and 2g.

3. Although slightly beyond the scope of this manuscript, the observed estrus cycle-dependent changes in connectivity (Fig. 2b–g) are striking and intriguing. I wish future studies hopefully explore the mechanisms underlying this remarkable plasticity and its potential functional roles.

We agree with the reviewer that this is a striking and intriguing phenomenon. We plan to follow up by performing a detailed morphological analysis to determine whether the loss of synaptic connections arises from presynaptic or postsynaptic changes.

4. The data in Fig. 2u–y largely reiterate findings from previous research (ref. 10). These could be moved to an ED Fig to streamline the main figures. Additionally, the data presented in Fig. 3j–u appear more closely related to the content of Fig. 2.

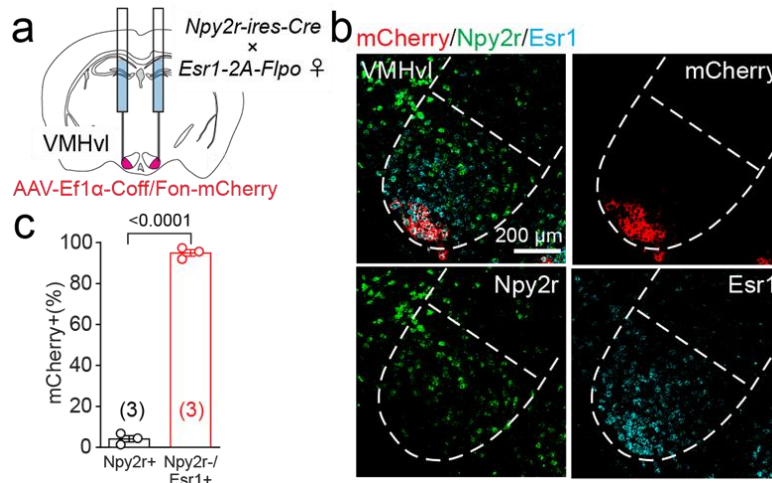
Thanks for the suggestion. We have moved Fig. 2u–y to Extended Data Figure 8. However, we have decided to keep Figure 3j–u in Figure 3, instead of moving it to Figure 2, as Figure 3 focuses on the PA^{Esr1}-VMHvl cell in vivo (a–i) and in vitro (j–u) changes during lactation.

5. Please clarify whether the animals used in ED Fig. 3 are the same as those used in Figs. 1e–i.

In Extended Data Fig. 3 (now Extended Data Fig. 5), the virgin females are different from the females used in Figs. 1e–i (now Figs. 1a–e). However, pup caring data (original Extended Data Fig. 3b and now Extended Data Fig. 5b) are from the same lactating females as shown in original Figs. 1e–i (now Figs. 1a–e).

6. The data in ED Fig. 6 are highly intriguing; however, it is necessary to histochemically validate that mCherry is indeed targeted to Npy2r–Esr1+ cells unless this has already been confirmed in previous studies.

We have now stained Npy2r and Esr1 in Esr1-Flp::Npy2r-Cre female mice that were injected with CoffFon-mCherry AAV into the VMHvl. We confirmed that the vast majority of mCherry-expressing cells were Npy2r- and Esr1+ in the VMHvl ($94.9 \pm 1.6\%$ cells from 3 animals) (**Reviewer Figure 12**). This new data is now shown in **Extended Data Fig. 9c, d**.



Reviewer Figure 12. Validation of selective targeting of Npy2r⁻ Esr1⁺ cells in the VMHvl

a, Experimental schematics.

b, Example images showing RNAscope in situ hybridization of mCherry (red), Npy2r (green), Esr1 (cyan) mRNA and their overlap in the VMHvl of an *Npy2r-ires-Cre::Esr1-2A-Flp* mouse injected with AAV expressing CoffFon-mCherry. Scale bars, 200 μm.

c, The percentage of mCherry cells expressing Npy2r⁺ and Npy2r⁻Esr1⁺ cells. Bars and error bars represent mean ± s.e.m. Numbers in parentheses indicate the number of animals. Statistical analysis was performed using unpaired t-test. All p values < 0.05 are indicated.

Referee #3:

This manuscript is a very interesting and elegant study that follows previous papers from this distinguished group. Here, they unravel how PA(Esr1) to VMHvl(Npy2r) as well as PVN(OXT) to PA connections in the maternal brain of lactating mice trigger maternal, but not general, aggression by combining knowledge (as significantly contributed by the Lin group) on brain circuits involved in male and female, i.e., maternal, aggression. This group has largely contributed to our understanding of aggression circuits, especially involving the VMH.

The authors use a huge range of elegant and highly targeted techniques, for which this group is well known for. The data is – in the combination - novel, and their presentation is of high quality; the chosen colors in the figures are a matter of personal taste. The study design and the statistical analyses are sound, I could not detect any flaws. The Discussion is clear and follows their own and other significant publications.

It was very elegant to include social interest and receptivity after viral manipulation. The fiber photometry experiment is another highlight as it shows how specific cells respond to aggression-provoking cues thereby leading to aggression.

We really appreciate the reviewer's positive and constructive feedback.

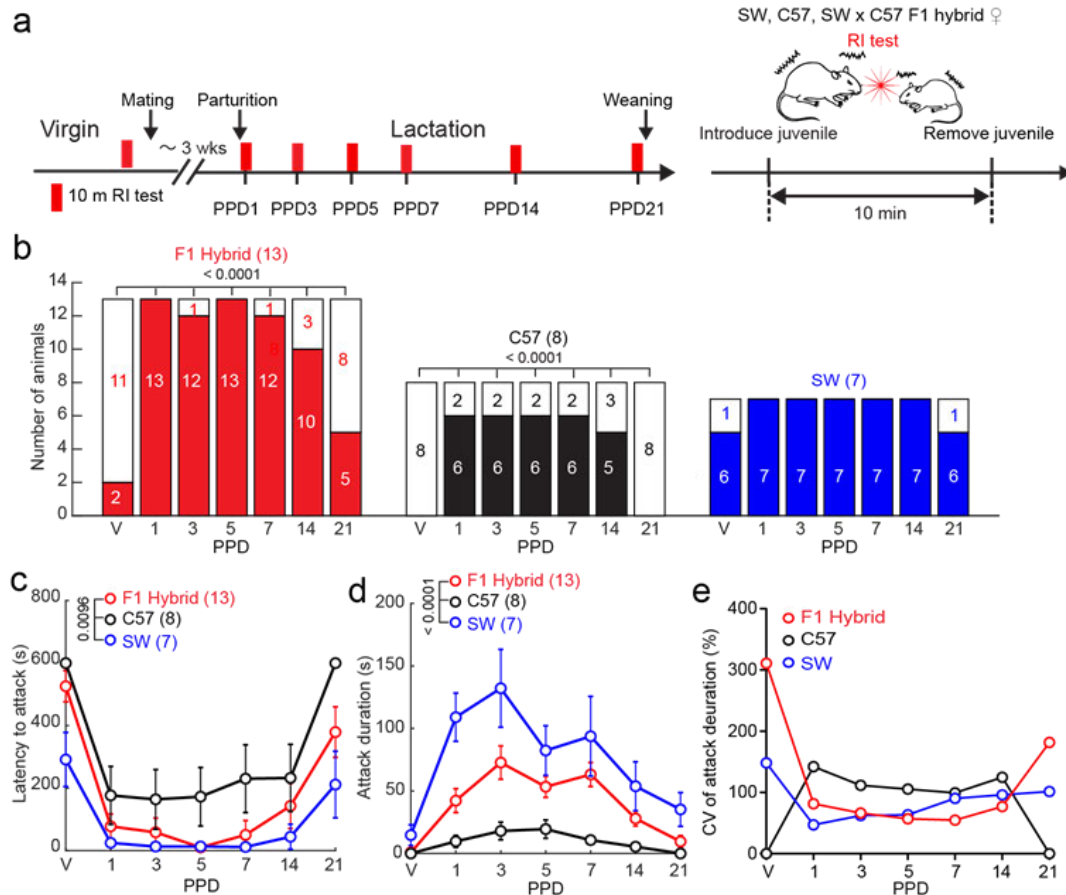
I have a few comments that could clarify some aspects or help to improve this work.

1. F1 generation of C57 x SW females showed reliable maternal aggression. As such, I always worry that the authors might have created a rather artificial mouse line where the circuits are different from WT mice. Thus, it would be convincing to give a final proof in WT mice.

In response to the reviewer's comment, we compared maternal aggression levels in C57, SW, and C57 x SW females. We found that the aggression level of C57 x SW females (measured by attack duration) falls between that of C57 and SW females (**Reviewer Figure 13a-c**). Moreover, the coefficient of variation (CV) of attack duration in the F1 hybrids is comparable to that of SW mice and lower than that of C57 mice (**Reviewer Fig. 13d**). These results indicate that C57 x SW females are not abnormally aggressive; their aggression lies within the normal range observed in laboratory mice.

We emphasize that C57 x SW females are wild-type mice, as they do not carry any engineered mutations. Their behavior may also more closely resemble that of wild mice, in which maternal aggression is robust and consistent. Due to artificial selection for docility, C57 mice—particularly C57 females—exhibit substantially reduced aggression compared to wild mice (Parmigiani, Palanza et al. 1999). We chose the C57 x SW F1 hybrid not only because of its more ethologically typical maternal aggression, but also because F1 hybrids are genetically identical: each gene is inherited as one allele from C57 and one from SW. Therefore, they are not more genetically variable than their parental strains. This hybrid strategy is well established and has been used in prior studies to generate genetically uniform transgenic mice with more robust behavioral

phenotypes, including aggression, compared to C57 mice (Golden, Jin et al. 2019, Sloin, Bikovski et al. 2022).



Reviewer Figure 13. Comparison of maternal aggression among C57 x SW F1 hybrids, C57, and SW mice.

a, Experimental timeline.

b, Number of females from different strains (C57 x SW F1 hybrids, C57BL/6N, and SW) that attacked a juvenile intruder during the 10-min RI tests across reproductive states. V, virgin.

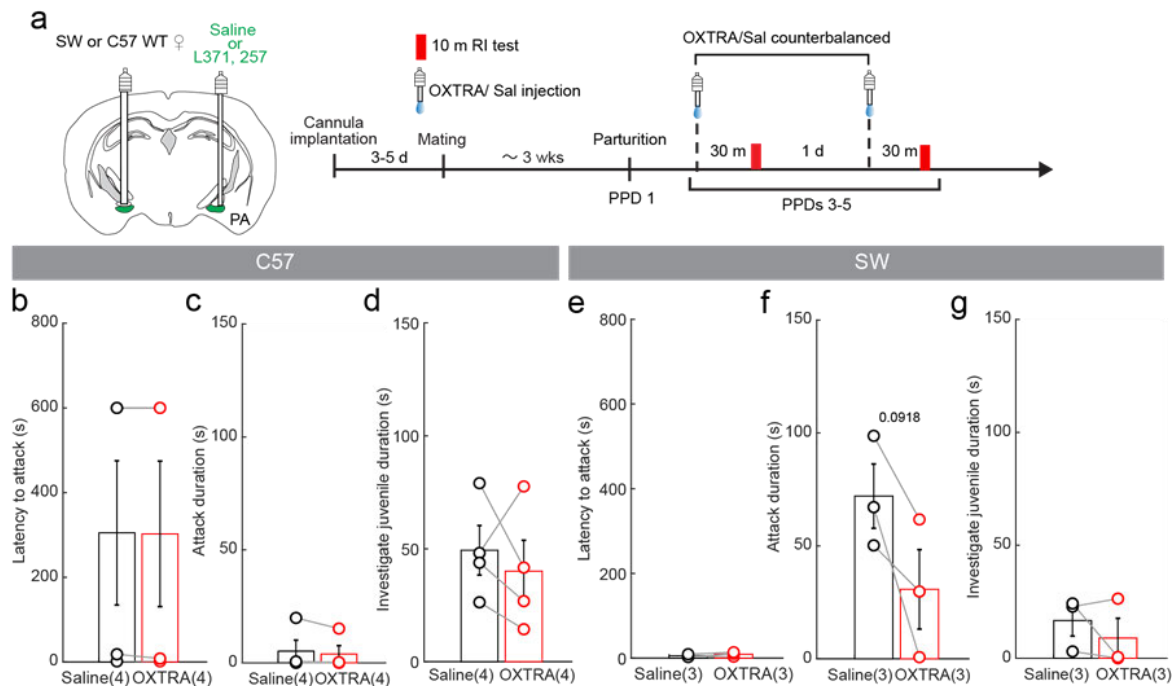
c,d, Attack latency (c) and duration (d) against a juvenile intruder in females from different strains (C57 x SW F1 hybrids, C57BL/6N, and SW). V, virgin.

d, The coefficient of variation (CV) of attack duration across days in females from different strains. V, virgin.

Bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number of animals. Statistical significance was determined using **(b)** Chi-square test, and **(c, d)** mixed-effect model with repeated measures followed by Bonferroni's multiple-comparison test. Post-hoc test results are not shown. See Supplementary Table 1, EDF 1k, 1l for statistical details.

While it is impractical to repeat all experiments in this study using SW and C57 mice, we performed a key experiment to verify that oxytocin modulates maternal aggression across strains. We injected an oxytocin receptor antagonist into the PA of SW and C57 lactating females and

found that this manipulation significantly reduced maternal aggression in SW mice (**Reviewer Fig. 14**). In C57 females, only 2 of 4 mice displayed brief attacks, limiting our ability to detect changes following OXTR antagonism (**Reviewer Fig. 14b, c**). The SW results are now presented in **Extended Data Fig. 11**. In addition, Liu et al. demonstrated that optogenetic activation of VMHv^{NPY2r} cells is sufficient to evoke aggression in virgin C57 females, a finding we replicated in C57 × SW F1 hybrid females (Liu, Eyring et al. 2022). Together, these results support the conclusion that the female aggression circuit—and its modulation by oxytocin—is conserved across mouse strains.



Reviewer Figure 14. Effect of OXTR antagonist in the PA on maternal aggression of C57 and SW females.

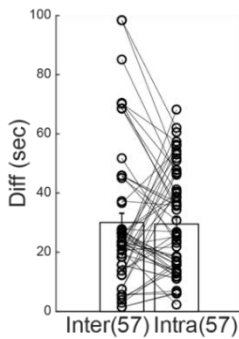
a, Experimental schematics and timeline.

b-g, Latency to attack (**b,c**), attack duration (**d,e**), and juvenile investigation duration (**d, g**) following injection of saline or L-371,257 hydrochloride, a potent OXTR antagonist (OXTRA), into the PA of C57 and SW females. OXTRA reduced attack duration but did not affect attack latency in SW females. Low maternal aggression in C57 females limits further reduction by OXTRA. All bars and error bars represent mean ± s.e.m. Numbers in parentheses indicate the number of subject animals. Statistical analysis was performed using paired using the Wilcoxon matched-pairs signed-rank test for (**b, c, g**) and paired t-test (**d-f**).

Did the authors make sure to test F1 females across different litters? How many litters have been used for the different mouse lines?

This is an important point. For each experiment, we used 1–7 litters, and whenever possible, animals from the same litter were included in both control and test groups. We compared maternal aggression levels within litters and across different litters and found no consistent litter effect—

that is, animals from the same litter did not show uniformly similar aggression levels (**Reviewer Fig. 15**). **Reviewer Table 1** summarizes the number of litters used in each experiment.



Reviewer Figure 15. Intra-litter and inter-litter maternal aggression variability.

No difference in attack duration between intra- and inter-litters. Intra-litter attack duration: average difference in attack duration between an animal and its littermates. Inter-litter attack duration: average difference between an animal and individuals from other litters in the same experiment. Numbers in parentheses indicate the number of animals. Bars and error bars represent mean $\pm$ s.e.m. Statistical analysis was performed using paired t-test.

Figure	Number	Non-Surgery		Control		Test	
		Litter	Animal	Litter	Animal	Litter	Animal
1	a-d	7	13				
	e-i			3	5	5	7
	j-p			4	6	6	7
3	a-i					4	7
4	j-m					4	10
	n-q			5	7	5	8
5	a-d			6	7	5	7
	e-i			4	6	3	5
	j-l					5	10
	m-p					3	6
EDF1	F1 hybrids	7	13				
	SW	1	7				
	C57	2	8				
EDF2	b-d	4	8				
	e-g	2	8				
	h-j	1	7				
EDF4	b-e					3	7
	f-l					4	7
EDF5	b			3	5	5	7
	e-i			2	6	5	7
EDF6	c, d			4	6	4	7
EDF7	d-f					3	3
EDF8	d, e					2	6
EDF10	b-e					4	8
EDF11	b-d					2	4
	f-g					1	3
EDF12	f-h					3	7
	j-l			3	5	5	6
EDF13	c-f			2	5	2	7

Reviewer Table 1. Summary of litters and animals per experiment. Non-surgery: animals without surgical procedures. Control/test: animals in respective experimental groups.

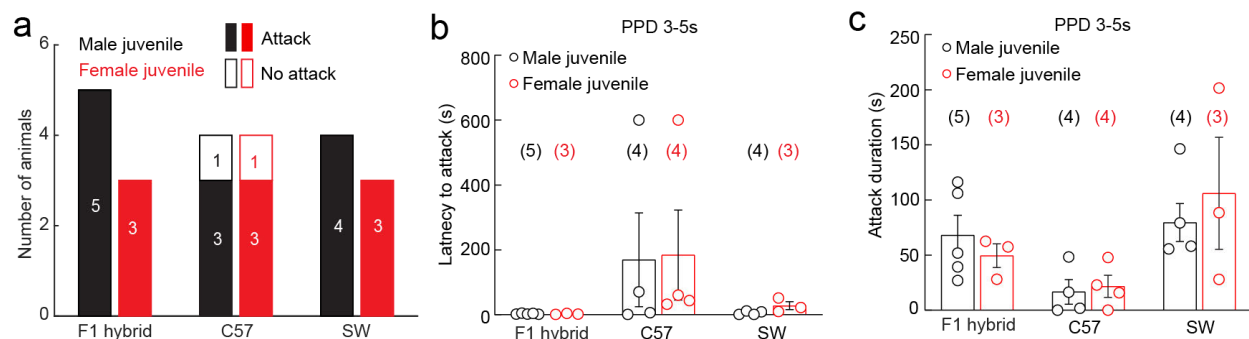
The change in maternal aggression intensity over the postpartum period as shown here in mice is very interesting and confirming previous mouse studies. Interestingly, such a decline in maternal aggression is not unique to mice as it has been shown for lactating rats as well (Caughey et al. 2011), hence being a rather general mechanism. That's probably to save the mothers' energy resources as soon as the pups become gradually more mobile – and being less in direct touch with the mother thereby being less of a positive stimulus to her.

We fully agree with the reviewer that the decline in maternal aggression as pups become mobile is a general phenomenon observed across many species. We have now incorporated the rat study mentioned by the reviewer into the Introduction (**Lines 39–41**).

In mice and rats, non-lactating females rarely attack conspecific intruders; however, aggression increases sharply one day after parturition, peaks during the first postpartum week, then gradually declines and essentially disappears by the end of lactation (Svare and Gandelman 1973, Caughey, Klampfl et al. 2011).

In the Results, the sex of the juvenile intruder should be mentioned. In the Methods, it is mentioned that male and female intruders have been used; did the authors detect any differences in the mothers' behaviors towards the intruders based on their sex?

We did not observe any difference in maternal aggression toward male versus female juvenile intruders (**Reviewer Figure 16**). This information has now been included in **Extended Data Figure 2k-m**.



Reviewer Figure 16. No difference in maternal aggression towards male and female juvenile intruders.

a, Number of females from different strains (F1 hybrids, C57BL/6N, and SW) that attacked male and female juvenile intruders. Fisher's exact test.

b, Lactating females across strains show no difference in attack latency toward male versus female juvenile intruders. (F1 hybrids, SW), Unpaired t-test; (C57), Mann–Whitney test.

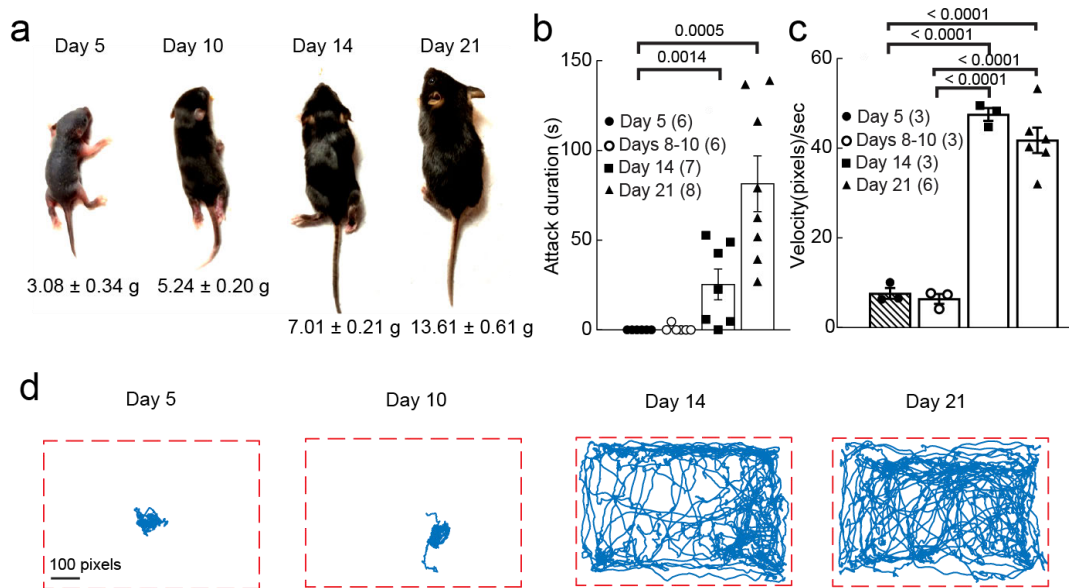
c, Lactating females across strains show no difference in attack duration toward male versus female juvenile intruders. Unpaired t-test.

Bars and error bars represent mean $\pm$ s.e.m. Numbers indicate the subject animal number.

Furthermore, I am a bit concerned regarding the definition of “juvenile”, which is starting at 15 days of age; at that stage the offspring are still considered as pups and are not weaned from their mothers. As mice are alloparental, it is even more surprising why lactating mouse mothers attack

those pups. In fact, I am wondering if the manipulations also made the mothers attack their own offspring? If so, this would suggest a different purpose that defending the offspring but rather a pup-attack circuit.

We thank the reviewer for raising this important question. In response, we examined the relationship between intruder age and maternal aggression. Consistent with a previous study (Svare and Gandelman 1973), we found that 10-day-old intruders never elicited attacks from mothers, whereas some 14-day-old intruders triggered low levels of aggression (**Reviewer Figure 17a, b**). The aggression level toward 21-day-old intruders was even higher (**Reviewer Figure 17a, b**). We also noted pronounced differences in mobility between 10- and 14-day-old pups: 10-day-old pups move very little, whereas 14-day-old pups continuously explore the cage, which may serve as an important trigger of female attack (**Reviewer Figure 17c, d**).



Reviewer Figure 17. The influence of intruder age on maternal aggression.

a. Representative images of mice on days 5, 10, 14, and 21. Typical weights (mean $\pm$ s.e.m) are indicated below the images. The weights are measured from 5-6 mice/age group.

b. Attack duration of PPD5 females toward intruders of different ages.

c. Movement velocity of intruder mice of different ages.

d. Representative movement trajectories of mice on days 5 (**d1**), 10 (**d2**), 14 (**d3**), and 21 (**d4**) during 10 minutes in a clean cage. Scale bar, 100 pixels. Red dash lines, outlines of the test cage.

All bars and error bars represent mean $\pm$ s.e.m. Numbers in parentheses indicate the number animals. Statistical significance was determined using (**b**) Kruskal-Wallis test with Dunn's multiple comparisons and (**c**) ordinary one-way ANOVA followed by Tukey's multiple-comparison test. All p values < 0.05 are indicated.

Importantly, the lactating females used in our study were tested between postpartum days 3 and 7, when maternal aggression is highest, and their own pups are easily distinguishable from 15-day-old intruders. After carefully reviewing our records, we found that we used 15-day-old

intruders only once in the PA^{Esr1}-VMHvl optogenetic activation experiment; in all other cases, the juvenile intruders were ≥ 19 days old. The use of the 15-day-old intruder was due to a limited testing window and the temporary unavailability of older juveniles. Across all experiments, the average age of juvenile intruders was 21.5 ± 1.3 days. We have now added the intruder age information for each experiment to the Methods section (**Lines 663, 684, 690, 698, 736, 751, and 761**).

We did not examine whether optogenetic stimulation of PA^{Esr1}-VMHvl terminals alters females' behavior toward their own pups, as all pups were routinely removed prior to intruder introduction.

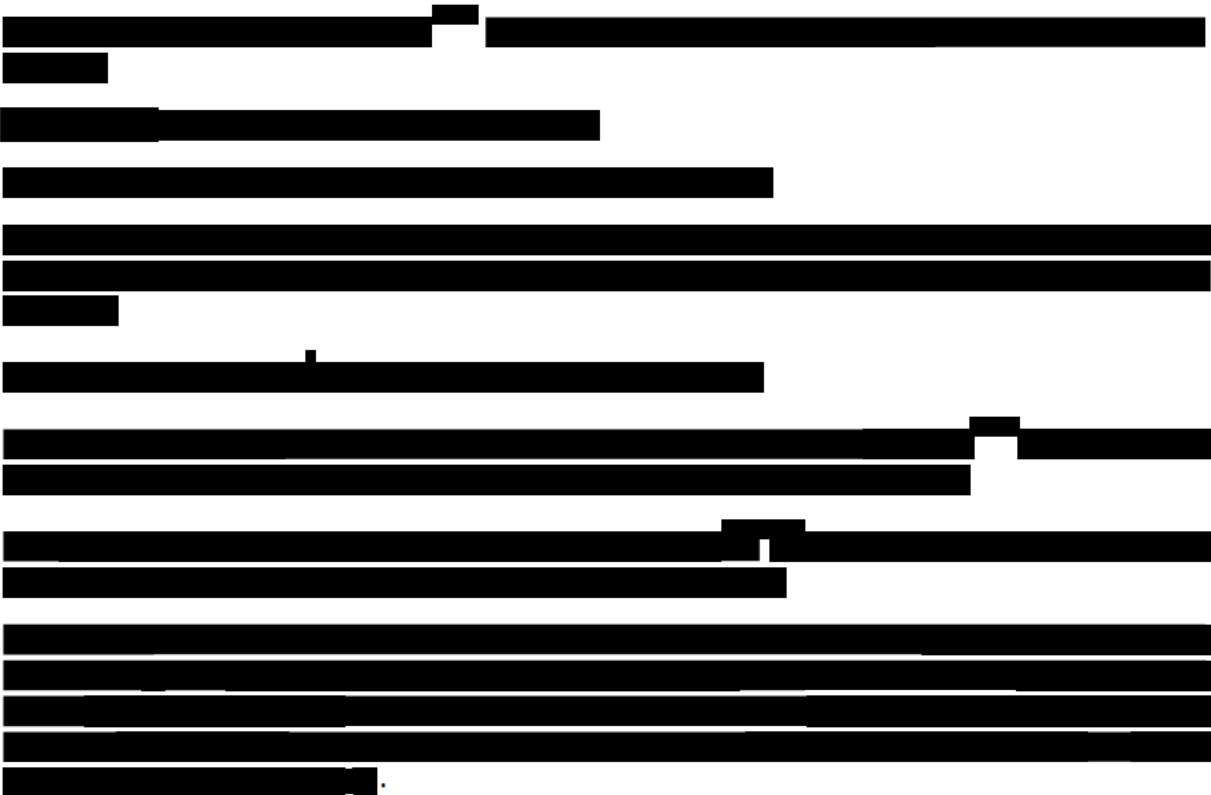
As the described pathway is only relevant for maternal aggression and cannot be stimulated in virgin females, potential differences in the expression of the investigated systems of NPY and ESR, including their receptors, need to be studied. Especially given that oxytocin signaling, which is increased in lactation (as has been shown also in the present study for the PA(Esr1)), can influence both systems – and vice versa.

As noted by the reviewer, activating the PA^{Esr1}-VMHvl pathway reliably evoked time-locked attacks in lactating females but not in virgin females. In contrast, direct activation of VMHvl^{Npy2r} cells induced robust attack behavior in both virgins and lactating females. These findings suggest that the PA^{Esr1}-VMHvl pathway is insufficient to activate VMHvl^{Npy2r} cells in virgin females. This conclusion is further supported by our in vitro recordings: stimulation of PA^{Esr1}-VMHvl terminals triggered spiking in only $\sim 15\%$ of VMHvl^{Npy2r} cells in virgins, whereas this proportion increased to $\sim 50\%$ in lactating females. We also showed that this heightened probability of spiking results from a combined effect of increased intrinsic excitability of VMHvl^{Npy2r} cells and strengthened connectivity between PA^{Esr1} and VMHvl^{Npy2r} cells.

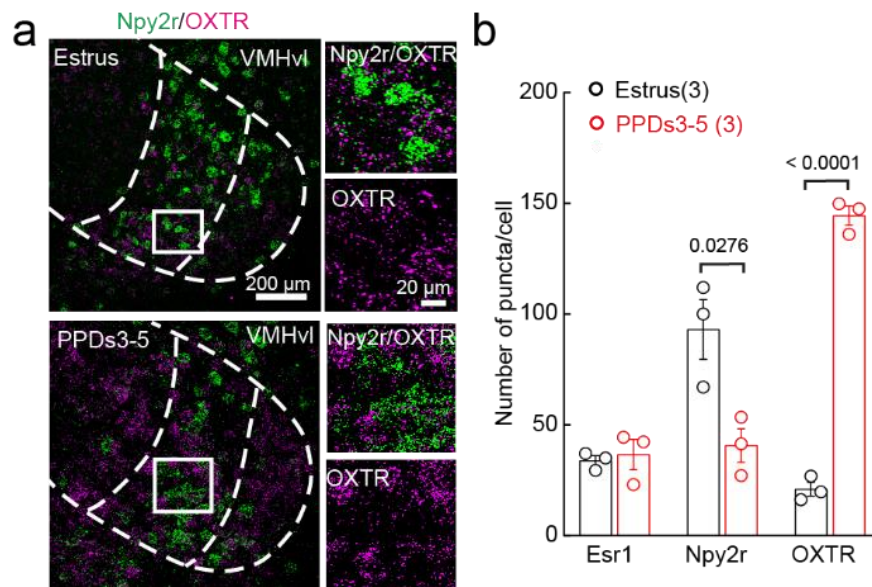
These physiological changes, measured in acute brain slices perfused for hours in ACSF containing no NPY or estrogen, are unlikely to reflect acute hormonal or neuropeptide modulation. Instead, we speculate that they arise from hormonal surges during pregnancy. [REDACTED]

[REDACTED]

[REDACTED]



Regarding the reviewer's specific question about *Esr1* and *Npy2r* expression, we quantified both markers in the VMHvl and found that *Esr1* expression is comparable between virgin and lactating females, whereas *Npy2r* expression decreases during lactation (**Reviewer Figure 19**; now included in **Extended Data Figure 12b**). This reduction is particularly interesting because *Npy2r* is a Gi-coupled receptor that typically suppresses neuronal activity upon ligand binding (Gammie, Auger et al. 2007). Thus, decreased *Npy2r* signaling may further contribute to the increased in vivo activity of VMHvl^{*Npy2r*} cells during lactation. Determining when NPY is released in the VMHvl and how it modulates maternal aggression will be an important direction for future research.



Reviewer Figure 19. *Esr1*, *Npy2r*, and *OXTR* expression in the VMHvl in virgin and lactating females.

a, Representative images showing *OXTR* (magenta) and *Npy2r* (green) mRNA in the VMHvl of estrus and lactating females. Right panels show enlarged views of the boxed areas. Scale bars, 200 μm (left), 20 μm (right).

b, Number of *OXTR*, *Npy2r* and *Esr1* mRNA puncta per corresponding *OXTR*-, *Npy2r*- and *Esr1*-expressing cell in the VMHvl of estrus and lactating females. Bars and error bars represent mean ± s.e.m. Numbers in parentheses indicate the number of subject mice. Statistical significance was determined using unpaired t-test. All p values < 0.05 are indicated.

It is very elegant to extend the studies to the oxytocin system in both PA and VMHvl. The findings suggest that oxytocin receptor signaling is more relevant in the PA than the VMH, at least in the specific cell populations investigated. Furthermore, the PVN as source of oxytocin on the PA is striking.

We thank the reviewer for their encouraging comments.

Please provide the number of mice used for the cannula implantation.

For the PA OXTR antagonist experiment in Figure 4, we used 10 animals in the saline group and 10 animals in the OXTRA group. For the VMHvl OXTR antagonist experiment in Extended Data Figure 12, we used 7 animals per group. For the PVN^{OXTR} cell stimulation combined with PA OXTR antagonist experiment in Figure 5, we also used 7 animals in both the saline and OXTRA groups. The subject numbers have now been indicated in all relevant figure panels.

As the aggression tests were performed in the home cage, please provide more information, i.e., were those standard mouse cages (supplier, product number, dimensions); such information would help to get a better idea of the overall conditions of the test.

The home cage used in our study was the Sealsafe Plus GM500 (39.1 cm × 19.9 cm × 16 cm). We have now added this information to the Methods section (**Lines 666-667**).

I might have missed this information, but at which time of day (or phase of the light dark cycle) have the experiments been performed? The only information I could find was "...we performed the RI tests at approximately the same time the day...".

All experiments were performed during the dark phase of the light-dark cycle (10 a.m. to 4 p.m.) of the animals. We have now added this information to the method (**Line 527**).

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Reviewer Responses:

Original comments in **blue**

Our responses in **black**

Referee #1

Comments on revised manuscript by Yamaguchi, Yan et al.

The revised manuscript addressed all of my comments. Therefore, I find the manuscript appropriate for publication in Nature with minor corrections. I would like to congratulate the authors on this major achievement linking maternal aggression and neuropeptide signaling.

We greatly appreciate the reviewer's support of our manuscript.

Minor points:

- The current manuscript has many typos for example:

o Line 55 "meidal"

We have changed "meidal" to "medial".

o Throughout the manuscript e.g. but not limited to line 192 to line 212: diestrous -> diestrus

o estrous: depends on the context - if meant as the specific phase of the estrous cycle, use estrus

We used estrous and diestrous as adjectives (e.g., estrous female, estrous cycle) and estrus and diestrus as nouns (e.g., in estrus). We will be happy to revise the manuscript to conform to the journal's preferred terminology.

o Figure 4a, 5s -> *Oxtr* (as in mRNA, not protein)

We have changed *Oxtr* to *Oxtr* and *Esr1* to *Esr1* in those figures to indicate that these are mRNA expression.

- Line 81 to 106 – I found the beginning part of the manuscript could be in the methods, to focus on the main conclusions rather than on technical points.

We have moved the part describing the female aggression towards different types of intruders and strain differences in female aggression (originally line 81-106) to Supplementary Note 1.

Referee #2

The authors have conducted a substantial number of additional experiments and data analyses that satisfactorily address all of my initial concerns. I now have no remaining issues. I appreciate their efforts and congratulate them on this significant milestone in behavioral neuroscience.

We thank the reviewer for their support of our manuscript.

Referee #3

The authors have revised their manuscript and replied to all my comments in a satisfactory manner. The work they put in is impressive. In my opinion, this study will make a highly valuable contribution how we see and understand maternal aggression.

We thank the reviewer for their support of our manuscript.