Sex-biased effects on hippocampal circuit development by perinatal SERT expression in CA3 pyramidal neurons

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Original submission

First decision letter

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MS TITLE: Sex-differential regulation of a critical period of hippocampal circuit development by transient SERT expression in CA3 pyramidal neurons

AUTHORS: Roberto De Gregorio, Galadu Subah, Jennifer Chan, Luisa Speranza, Xiaolei Zhang, Aarthi Cahn, Li Shen, Ian Maze, Patric K Stanton, and Jiying Y Sze

I have now received the reports of three referees on your manuscript and I have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, all the referees are enthusiastic about your work, but they also have significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy to receive a revised version of the manuscript. Your revised paper will be re-reviewed by the original referees, and its acceptance will depend on your addressing satisfactorily all their major concerns. Please also note that Development will normally permit only one round of major revision.If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee’s comments, and we will look over this and provide further guidance.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this study, De Gregorio et al. investigate the role of serotonin uptake transporter (SERT) on the maturation of hippocampal circuitry during early postnatal development, using molecular, neuronal, circuit-level and behavioural approaches. The authors first characterise SERT expression in developing hippocampus, as well as the projection patterns of SERT+ CA3 neurons. They then generate SERT-PyramidΔ KO mice in which SERT is ablated in both the CA3 region and the mPFC. The density of CA1 neurons, which are targeted by CA3 neurons, is unchanged in these mice, but they exhibit reduced numbers of (immature) spines at P16 and increased numbers of mature spines as adults. Correspondingly, LTP magnitude in adult female, but not male, SERT-PyramidΔ mice at CA3-CA1 Schaffer collateral synapses, whereas LTD magnitude is reduced in both sexes. The authors characterise SERT-PyramidΔ mice in several assays to address potential behavioural effects. They find that (a) male (but not female) SERT-PyramidΔ exhibit increased immobility time in forced swim test and spend less time in open arms of elevated plus maze, (b) SERT-PyramidΔ mice show a reduced preference for a novel mouse in a social interaction test, and (c) female KO mice show less freezing in contextual fear conditioning test. Finally, De Gregorio et al. characterize hippocampal transcriptional profiles from SERT-PyramidΔ and control animals, finding only subtle, sex-shared and sex-specific effects. DEGs were enriched for ASD-associated genes, and Kras and Braf in particular (which have been shown to regulate neural circuit development and function) are reduced in male SERT-PyramidΔ mice.

This comprehensive study makes an important contribution to our understanding of the role of SERT in early hippocampal development. It is overall well executed with appropriate controls. My comments are therefore minor in nature, and I would recommend this study for publication in Development provided that the authors address the following points.

Comments for the author

Main points
• Fig. 1: What fraction of CA3 neurons is SERT+?
• Fig. 2: The authors mention that 5-HT staining in control (SERT-flox/flox)
animals was only present in glutamatergic neurons when pups were treated with clorgyline. Were the SERT-PyramidΔ (Fig. 2b, bottom row) also treated with clorgyline? Also, the 5-HT staining seems to be excluded from neuronal cell bodies
(top row, 2nd panel) – a NeuN or DAPI counterstain would be helpful to address this.
• Fig. S2: coordinates should be indicated from Bregma, as common, not from the tip of the brain
• Fig. 5: An important caveat of these behavioural experiments is that SERT expression is abolished in both hippocampus and mPFC in SERT-PyramidΔ mice.
Although mPFC SERT+ neurons do not seem to project to hippocampus, lack of SERT in mPFC might in fact underlie some or all of the observed behavioural changes. This could in principle be addressed by brain area specific delivery of Cre recombinase via AAV injections into SERT flox/flox mice. Such an approach would have the added benefit of being able to target the affected CA3 neurons for anatomical and functional interrogations. I am not suggesting that the authors necessarily do this (and the authors address this in the Discussion), but this very important limitation should be more prominently highlighted in the results section as well.
• Could the authors please discuss how the observed behavioural alterations are related to Autism Spectrum Disorders? This is currently not very clear.

Minor points
• Please indicate what the staining is in fluorescent image panels of most figures. Although this information can be found in figure legends, it could be much easier to understand the figures.
• Language / grammar needs editing in several places, e.g.:
-p4: duplication/grammar: “How sex-differential neural circuits are established during normal development, when and how in the developmental course sex-dependent circuit derailment may arise in diseased brain may arise are currently unknown”
-p4 wording: “Patricia Gaspar, subsequently by others and ourselves, identified in rodents SERT expression…” --> Gaspar et al. identified […] in the rodent thalamus and this was subsequently confirmed by others, including ourselves.

-p5: “Many human brain developmental processes in mid-fetal gestation occur in the first two postnatal weeks in mice” --> better: correspond to the first two postnatal weeks in mice
-p5: “We have developed a SERTflox/flox-Cre system in mice and demonstrated…” --> we have developed SERTflox/flox-Cre mice
-p7: “Consistent with CA3c pyramidal neuron backprojection” --> CA3c?
-p9: “About 85% of adult DG granule cells arrive the” --> at the?
-p9: “We tested this idea by examining postnatal development of a GFP reporter driven by a Thy1 promoter cassette” --> […] development of neurons labelled by a GFP […]?
-p10: “that might be overcame in adult hippocampus.” --> overcome
-p14: “transcriptomes of ASD, schizophrenia and depression brain tissues” --> transcriptomes from brain tissues from patients diagnosed with […]
-p14: define ASD

Reviewer 2

Advance summary and potential significance to field

This manuscript describes a comprehensive investigation of a role for developmental SERT expression in pyramidal neurons in axon growth, synapse formation, and synaptic function, with attention to hippocampal development and sex-specific contributions. The questions asked are significant, since little is known about this particular SERT mechanism, and its contribution to sex-specific presentation of developmental disorders is of import and is well described by the authors. The experimental design is sophisticated, and rigorous attention was paid to the cell-specificity and functional verification of SERT knockout in CA3 pyramidal neurons. In general, I believe this paper to be of very high merit however a few substantive issues need to be addressed.

Comments for the author

Abstract:
More directional language would be very helpful to frame the findings of the reported studies (rather than using terms like “differentially regulates” and “differentially alterations”). Additionally, it is not appropriate to describe the manipulation of the study as the knockout of hippocampal pyramidal SERT, since as stated by the authors in the manuscript, pyramidal neurons in other brain areas were affected as well.
Results:
-I do not believe that the use of t-tests within each sex is appropriate, given the focus on sex-specific effects of SERT knockdown. In order to conclude that effects were sex-specific, 2-way ANOVAs are required with sex as a factor.
-Quantification of SERT-expressing neurons over development should be provided (Fig 1), in addition to the images shown.
-The sex of the mice assessed for the time course of SERT expression and tracing studies was not reported. Were there sex differences in these projection patterns? This is important information given that a focus of the investigation was sex differences in the functional consequences of SERT expression
-The behavior data are interesting, however it is very unfortunate that the fear conditioning paradigm was not extended to measures that might help tease apart reliance on hippocampal LTP versus LTD (e.g. fear retrieval).
Minor: Grammar should be edited throughout the manuscript

Reviewer 3

Advance summary and potential significance to field

This manuscript by De Gregorio and colleagues makes the interesting observation that the regulation of the serotonine uptake transporter (SERT) may be implicated in the development of the hippocampal circuit during a narrow time window of postnatal development. This developmental role of SERT is particularly impacting the CA3-CA1 communication due to the specific transient expression of SERT in CA3 cells (Fig. 1). The authors show that a disruption of

SERT expression in these cells (Fig. 2): (1) alters spinogenesis in a subset of CA1 pyramidal neurons with male and females showing less thin and more mushroom spines in third order dendrites of juvenile and adult mice, respectively (Fig 3); (2) leads to sex-biased impairment of CA1 LTP and LTD in the adult CA1 (Fig. 4); (3) has sex-biased behavioral consequences (Fig. 5); (4) causes sex-biased gene expression at P7. Previous work had already shown that changes in serotonin homeostasis during critical periods of development can impact specific circuits and lead to major consequences in the adult. However, this set of observations is novel, intriguing and potentially important. They cover several aspects of hippocampal function and span many scales of analysis, from genes to circuits and behavior.

Comments for the author

Maybe because of this wide scope of analysis, the reader is left with the impression of a catalog of almost preliminary but very interesting datasets without any obvious link between them. The authors do not really try relating these different scales of analysis, even in the discussion but never the less make very broad and strong statements in their abstract and introduction that should be down-tuned. For example, I am not convinced that this manuscript identifies “a temporal-specific modulatory mechanism causing sex-biased cognitive and behavioral impairments” because the data presented here does not reach a mechanistic level of understanding. The basic early steps of circuit dysfunction during development are overlooked, including the fate of the CA3 neurons and their wiring to their postsynaptic CA1 targets. Also, with the genetic analysis done in Fig. 6, we do not know whether the effect of SERT and modulating serotonin levels is trophic and activity-dependent or related to gene regulation. Overall, this paper is interesting but instead of this wide panel of observations, I would suggest maybe narrowing slightly its focus with a better analysis of the remaining observa

Related to figure 1:
The authors use in situ hybridization of SERT mRNA from P3 to P14 to support the claim that SERT is expressed in a subset of CA3 pyramidal neurons (Fig. 1a). This statement should be supported by a more quantitative and anatomical (which part of CA3, a, b, c? which CA3 sublayer? Position along te dorso-ventral axis?, etc.) description of the subset of CA3 cells expressing SERT mRNA (instead of unessential quantifications such as the one displayed in Fig. 1f). Next, the authors use a SERT-Cre mouse to study further these cells, however, these experiments are difficult to interpret. Indeed, the fact that tdTomato expression increases with age may be independent from the actual number of cells expressing SERT at the time of analysis. Td Tomato is only indirectly reporting SERT expression. For example, tdTomato will still be expressed at a time when cells that once expressed SERT are longer expressing it. Instead, the developmental timeline of SERT expression should be done with immunolabellings for SERT (such as Fig. 1b). In addition, those immunolabellings should be displayed at lower magnification (as in Fig. 1b left image). One would need to know the fraction of tdtomato cells that are co-labelled with SERT. Related to the rest of the manuscript, it would be important to know whether the development of SERT is similar in males and females. Still, I do not see the point of the hub and ctip 2 labellings as CA1 and CA3 can be more easily identified using anatomical criteria.
One can observe a strong td Tomato labeling in the lacunosum moleculare (1b left),
which is probably related to the expression of the reporter in axonal tracts impinging onto this layer. This could indicate significant SERT expression in extra-hippocampal long-range inputs, the two more likely being ECL3 or the Ventro Medial thalamus. The authors may need to check this as it may complicate the interpretation of their LOF findings.

Related to figure 3:
The authors examine the impact of SERT LOF in CA3 pyramids on CA1 principal cells using a Thy1-GFP mouse line as in Deguchi et al 2011. This raises several concerns. First, it is surprising that the authors do not investigate the direct effects of SERT LOF on CA3 neurons themselves (anatomy, excitability connectivity, etc) but instead jump one synapse away from the initial insult.
Also, the mouse line used to analyzed CA1 pyramids should be specified. Indeed Deguchi et al use two different Thy1-GFP mouse lines which both label e subpopulation of CA1 cells, that might represent the earliest principal neurons in the hippocampus. The possible specifity of the subpopulation analyzed here should be taken into account. The authors need to consider the possibility that they may be analyzing the effect of SERT LOF on a subset of cells with specific developmental trajectory. According to Deguchi et al, these cells should preferentially connect to

CA3 neurons with a similar developmental time line: are the CA3 cells expressing SERT located in the early born subregions of CA3 (i.e. CA3a and b)?
The number of Thy1-GFP cells is not different between control and SERT pyramids but the number of cells seems to vary a lot between age groups (eg. Around 1500 at P16 compared to 400 at P6), how can the authors explain this? Have the cells been sampled in the same location along the dorso-ventral and proximo-distal axes?

Related to Fig. 4:
The authors show a very strong sex-dependent effect of SERT LOF on LTP/LTD with a blockade of LTD in both sexes and a boost of LTP in females. How can this major finding be linked to the previous analyses? How about LTP/LTD in juvenile mice that display less thin spines? Is expression of SERT and its localization the same in males and females?

Related to fig. 6: the authors observe subtle changes in expression levels of genes in SERT LOF experiments at P7. They chose to analyze the datasets with p<0,05, which represents the lowest significance level. Why not focus on the smallest list of DEGs obtained at p<0,005? Would the conclusions be different? Why choose P7 since it corresponds to the peak of SERT expression? Maybe adult or juvenile analysis would have made more sense if one wants to relate this to the observed juvenile/adult modifications in CA1 anatomy and plasticity.

Minor:
“we show SERT expression in CA3 pyramidal neurons coinciding with hippocampal circuit establishment”: what is meant by “hippocampal circuit establishment”?
tions. Besides these general comments, my main concerns are detailed below:

First revision

Author response to reviewers' comments

De Gregorio et al. DEVELOP/2022/200549
We thank the editor and the three reviewers for detailed evaluation of our manuscript and many helpful suggestions. In this revised manuscript, we present data from new experiments and revised text following referees’ suggestions. Please see our point-to-point response below.

Referee #1
This comprehensive study makes an important contribution to our understanding of the role of SERT in early hippocampal development. It is overall well executed, with appropriate controls. My comments are therefore minor in nature, and I would recommend this study for publication in Development provided that the authors address the following points.

Response: We thank the reviewer for the evaluation of our work.

Main points:
Figure 1. What fraction of CA3 neurons is SERT+?

Response: We thank the reviewer for bringing up this important characterization question. We have carried out new experiments to assess the fraction of SERT-expressing neurons in CA3 by quantitating % of SERT-Cre dependent tdTom reporter-expressing neurons over DAPI-labeled cell nuclei in the CA3 pyramidal layer at four developmental stages (E17.5, P0, P3 and P6) in both males and females, and data are presented in Fig 1D.

Figure 2. The authors mention that 5-HT-staining in control (SERT-flox/flox) animals was only present in glutamatergic neurons when pups were treated with clorgyline. Were the SERT-PyramidΔ (Fig. 2b, bottom row) also treated with clorygline?

Response: Yes, SERTPyramidΔ mice and control littermates were treated and processed in parallel in every experiment throughout our studies in a manner described in the Materials & Methods.

Previous studies have demonstrated robust expression of monoamine degradation enzymes including MAOA in SERT-expressing glutamtergic neurons (Solza-Reilly et al. Mol. Psychiatry, 2019). In this experiment, SERTPyramidΔ pups and control littermates were treated with either the MAOA inhibitor clorygline or vehicle saline, to demonstrate: a) in control pups, these glutamatergic neurons took up extracellular 5-HT and effectively degraded it and the degradation was blocked by clorygline, supporting the notion that CA3 SERT serves to limit extracellular 5-HT levels, and b) In SERTPyramidΔ pups treated with clorgyline, 5-HT staining can be detected in the thalamic glutamatergic neurons (a positive control for clorgyline efficacy in the treated pups, please see Fig 2B), but not in the CA3 and mPFC pyramidal neurons, indicating that SERTPyramidΔ selectively abolished 5-HT uptake by these CA3 and mPFC pyramidal neurons.

Also, the 5-HT staining seems to be excluded from neuronal cell bodies (top row, 2nd panel) – a NeuN or DAPI counterstain would be helpful to address this.

Response: We thank the reviewer for the keen observation and the suggestion. We did perform DAPI counterstain for the 5-HT immunostaining, and the corresponding images showing 5-HT immunostaining and DAPI overlay have been added to Fig 2B. SERT is highly expressed in axonal terminals of those glutamatergic neurons, and following clorygline treatment 5-HT immunoreactivity may be detected in the cell body and their neurites (Chen et al. Cell Reports, 2015). In the CA3, SERT-expressing CA3 pyramidal neuron axon collaterals pass through the midline to innervate contralateral hippocampus (please see Fig. 1E), and 5-HT staining may label SERT-expressing CA3 neurons and their neurites in the ipsilateral hippocampus and SERT-expressing CA3 pyramidal neuron axons from contralateral hippocampus.

Fig. S2: coordinates should be indicated from Bregma, as common, not from the tip of the brain.

Response: We have justified the anatomical positions of the serial sections in Fig. S4, according to their Bregma coordinates.

Fig. 5: An important caveat of these behavioural experiments is that SERT expression is abolished in both hippocampus and mPFC in SERT-PyramidΔ mice. Although mPFC SERT+ neurons do not seem to project to hippocampus, lack of SERT in mPFC might in fact underlie some or all of the observed behavioural changes. This could in principle be addressed by brain area specific delivery of Cre recombinase via AAV injections into SERT flox/flox mice. Such an approach would have the added benefit of being able to target the affected CA3 neurons for anatomical and functional interrogations. I am not suggesting that the authors necessarily do this (and the authors address this in the Discussion), but this very important limitation should be more prominently highlighted in the result sections as well.

Response: We agree and thank the reviewer for the thoughtful comments. Indeed, all the behaviors we tested with SERTPyramidΔ mice are known to be regulated by both mPFC and hippocampus. SERT may coordinately regulate synaptic circuit assembly at the hippocampus by CA3 SERT and at mPFC SERT-expressing pyramidal neuron projection target regions (e.g. VTA and amygdala) to influence the behavior. We have now further emphasized this fact in the Results and Discussion. To date, however, cell-specific knock-in Cre mouse lines remain the most robust means to study the transient SERT expression (E17 to P10) in the pyramidal neurons, as delivering Cre recombinase via AAV injection after hippocampus formed did not allow sufficient time for propagating Cre to ablate the SERT expression in time. We have now clearly stated that phenotypes we have observed in the SERTPyramidΔ mice could serve as a basis for future studies to elucidate molecular mechanisms in the brain regions that are coordinately regulated by SERT-expressing mPFC and CA3 pyramidal neurons during circuit assembly.

Could the authors please discuss how the observed behavioural alterations are related to Autism Spectrum Disorders? This is currently not very clear.

Response: We have revised the Discussion to suggest the implications of our findings to autism and related neurodevelopmental disorders.

Minor points

Please indicate what the staining is in fluorescent image panels of most figures. Although this information can be found in figure legends, it could be much easier to understand the figures.

Response: We have added the staining in fluorescent images throughout the manuscript.

Language/grammar needs editing in several places.

Response: We have edited the entire manuscript. The term “CA3c pyramidal neuron backprojection” was proposed by Helen E. Scharfman, as she identified that CA3c pyramidal neuron collaterals innervate the dentate gyrus in addition to their “forwardprojection” to CA1 (Scharfman HE, Progress in Brain Res. 2007). SERT-expressing neurons are enriched in CA3c (please see Fig. 1D). We have revised the sentence to make it clear.

Referee #2
This manuscript describes a comprehensive investigation of a role for developmental SERT expression in pyramidal neurons in axon growth, synapse formation, and synaptic function, with attention to hippocampal development and sex-specific contributions. The questions asked are significant, since little is known about this particular SERT mechanism, and its contribution to sex-specific presentation of developmental disorders is of important and is well described by the authors. The experimental design is sophisticated, and rigorous attention was paid to be the cell-specificity and functional verification of SERT knockout in CA3 pyramidal neurons. In general, I believe this paper to be of very high merit, however a few substantive issues need to be addressed.

Response: We thank the reviewer for thoughtful evaluation of our work and appreciating our experimental design.

Abstract: more directional language would be very helpful to frame the findings of the reported studies (rather than using terms like “differentially regulates” and “differentially alterations”. Additionally, it is not appropriate to describe the manipulation of the study as the knockout of hippocampal pyramidal SERT, since as stated by the authors in the manuscript, pyramidal neurons in other brain areas were affected as well.

Response: We have revised the Abstract and text to clearly describe our findings. The reviewer is correct and as shown Fig 2A and Fig. S1, SERT is expressed in subsets of pyramidal neurons in two brain regions (CA3 and mPFC), and SERTPyramidΔ ablates SERT expression in both the regions. We have stated this fact explicitly throughout the manuscript.

Results:
I do not believe that the use of t-tests within each sex is appropriate, given the focus on sex-specific effects of SERT knockdown. In order to conclude that effects were sex-specific, 2-way ANOVAs are required with sex as a factor.

Response: We thank the reviewer for the suggestion. We have analyzed the datasets with 2-way ANOVA, for experiments where samples and animals were processed and analyzed in parallel (behavioral tests and RT-qPCR). For both FST and EPM, we observed a statistically significant genotype effect, with post-hoc analyses indicating significant (FST) and a strong trend (EPM) of the deficits only in SERTPyramidΔ males. For the Open Field mean distance, there was a trend for sex X genotype interaction $P = 0.0566$, with post-hoc analyses indicating significant deficits only in SERTPyramidΔ females. For RT-qPCR analyses, there was a significant sex X genotype interaction for both Kras and Braf expression levels in the hippocampus age P7, with post-hoc analyses showing a significant change only in SERTPyramidΔ males. Detailed 2-way ANOVA results are presented in Table S1 and discussed in the text.
As now indicated more clearly in the text, sex-biased effects by perinatal SERT expression in the pyramidal neurons were not expected. Therefore, several earlier experiments analyzed males and females independently, including spine morphometric analyses, electrophysiology and western blot. To avoid the possibility to detect in-between sex difference due to experimental variations from sample collections, storage and processing, we performed t-test comparing SERTPyramidΔ vs. control littermate mice, since some published studies also utilize t-test to evaluate sex differences

in gene functions among males or among females (for an example, please see Labonte et al. Nature Medicine, 2017).

Quantification of SERT-expressing neurons over development should be provided (Fig 1), in addition to the images shown.

Response: We have quantified the percentage of SERT-expressing neurons in the CA3 pyramidal layer at the 4 developmental stages corresponding to the images shown in Fig 1C and data are presented in Fig. 1D.

The sex of the mice assessed for the time course of SERT expression and tracing studies was not reported. Were there sex differences in these projection patterns? This is important information given that a focus of the investigation was sex differences in the functional consequences of SERT expression.

Response: We agree this is important information. We did examine the time course of SERT expression and axon innervation fields of SERT-expressing CA3 neurons in both males and females – we observed no appreciable differences between the two sexes. In addition, we also traced the innervation fields of SERT-expressing mPFC pyramidal neurons in both males and females (Fig. S4) and observed no difference between the two sexes. The number of female and male mice in which SERT-expressing mPFC or CA3 pyramidal neuron projections we traced have been added. In order to statistically compare the time course of SERT expression in males vs. females, brains from the two sexes need to be collected, processed and analyzed in parallel such that every male is controlled by at least one female littermate and vice versa. To address the reviewer's question, we have carried out new experiments to quantify SERT-expressing neurons in the CA3 pyramidal layer in males and females at 4 developmental stages; data are presented in Fig. 1D and detailed statistical analyses results are presented in Table S1.

The behavior data are interesting, however, it is very unfortunate that the fear conditioning paradigm was not extended to measure that might help tease apart reliance on hippocampal LTP versus LTD (e.g. fear retrieval).

Response: We performed a range of behavioral tests to identify which behavioral paradigms may be sensitive to developmental SERT expression in the hippocampal and mPFC pyramidal neurons. As now explained more clearly, all the tested behaviors including the fear conditioning rely on complex neural circuits not exclusively regulated by the hippocampus and we hypothesize that SERT expression in the pyramidal neurons is positioned to coordinate 5-HT signaling during circuit assembly in the hippocampus, cortex and their subcortical target regions. We have revised the Discussion to state the purpose of the current studies and the limitations, and suggest that behavioral impairments we have identified from the current studies may provide experimental paradigms for future investigations into SERT downstream mechanisms that function coherently during neural circuit assembly in the hippocampus, cortex and subcortical brain regions to shape behaviors.

Minor: Grammar should be edited throughout the manuscript.
Response: We have edited the entire manuscript.

Referee #3
This manuscript by De Gregorio and colleagues makes the interesting observation that the regulation of the serotonin uptake transporter (SERT) may be implicated in the development of the hippocampus during a narrow time window of postnatal development. This developmental role of SERT is particularly impacting the CA3-CA1 communication due to the specific transient expression of SERT in CA3 cells (Fig. 1). The authors show that a disruption of SERT expression in these cells (Fig. 2): (1) alters spinogenesis in a subset of CA1 pyramidal neurons with males and females showing less thin and more mushroom spines in third order dendrites of juvenile and adult mice, respectively (Fig. 3); (2) leads to sex-biased impairment of CA1 LTP and LTD in the adult CA1 (Fig. 4); (3) has sex-biased behavioral consequences (Fig. 5); (4) causes sex-biased gene expression at P7. Previous work had already shown that changes in serotonin homeostasis during critical periods of development can impact specific circuits and lead to major consequences in the adult. However,

this set of observations is novel, intriguing and potentially important. They cover several aspects of hippocampal function and span many scales of analysis from gene to circuits and behavior.

Response: We appreciate the thorough review of our work and thoughtful comments.

Maybe because of this wide scope of analysis, the reader is left with the impression of a catalog of almost preliminary but very interesting datasets without any obvious link between them. The authors do not really try relating these different scales of analysis, even in the discussion but never the less make very broad and strong statements in their abstract and introduction that should be down-tuned. For example, I am not convinced that this manuscript identified “a temporal-specific modulatory mechanism causing sex-biased cognitive and behavioral impairments” because the data presented does not reach a mechanistic level of understanding. The basic early steps of circuit dysfunction during development are overlooked, including the fate of the CA3 neurons and their wiring to their postsynaptic CA1 targets. Also, with the genetic analysis done in Fig. 6, we do not know whether the effect of SERT and modulating serotonin levels is trophic and activity dependent or related to gene regulation. Overall, this paper is interesting but instead of this wide panel of observations, I would suggest maybe narrowing slightly its focus with a better analysis of the remaining observations. Besides these general comments, my main concerns are detailed below.

Response: This is the first characterization of the biological roles of SERT expression in the pyramidal neurons for hippocampal development. We feel that the systematic genetic, anatomical, circuit and behavioral analyses are necessary to establish that the transient SERT expression during this developmental stage is essential for achieving normal hippocampal synaptic circuitry in adulthood and behavior. Following reviewer’s question, we have confirmed that SERT is not required for general cell fate specification of the CA3 SERT-expressing neurons (Fig. S3D). Previous studies have also demonstrated that SERT is not required for cell fate specification of the glutamatergic thalamic- and mPFC-SERT-expressing neurons and their gross axon/dendrite growth (Chen et al., Cell Reports, 2015, Solza-Reilly et al. Mol. Psychiatry, 2019). These findings are consistent with the notion that regulations of cortical neuronal cell identity and their functional maturation process may be mechanistically separable (Rakic et al., 2009; Cadwell et al., 2019).

An important aspect of our work relates to the fact in human brain multiple high-confidant autism risk genes are specifically co-enriched in midfetal cortex, coinciding with the SERT expression in cortical glutamatergic neurons (Willsey et al. Cell 2013; Voineagu et al. Nature 2011Nowakowski et al. Science, 2017). Although alterations in midfetal development has been implicated as an etiological origin for autism and related neurodevelopmental disorders, very little is known about the biological consequence of disrupting any of the risk factors during this developmental time window, in part because those risk genes are continuously broadly expressed throughout the brain. Our studies identified a transient SERT expression in the pyramidal neurons in the CA3 and mPFC specifically during circuit development and this developmental SERT function is essential for normal synaptic plasticity in the hippocampus and cognitive behaviors. As many developmental processes in human midfetal brain correspond to the first two postnatal weeks in mice, we feel that the series of phenotypes resulting from disruption of the transient SERT expression in the pyramidal neurons in our mouse model will provide a basis for future elucidation in our lab and others of the fundamental mechanisms of neural circuit maturation and the function of those disease-associated risk factor during functional circuit assembly in normal brain and disease. We have revised the Abstract, Introduction and Discussion to emphasize our data. We have further improved the manuscript to address the reviewer’s suggestions as blow.

Related to figure 1: The authors use in situ hybridization of SERT mRNA from P3 to P14 to support the claim that SERT is expressed in a subset of CA3 pyramidal neurons (Fig 1a). This statement should be supported by a more quantitative and anatomical (which part of CA3, a,b,c? which CA3 sublayer? Position along te dorso-ventral axis, etc.) description of the subset of CA3 cells expressing SERT (instead of unessential quantifications such as the one displayed in Fig 1f). Next, the authors use a SERT-Cre mouse to study further these cells, however, these experiments are difficult to interpret. Indeed, the fact that tdTomato expression increase with age may be independent from the actual number of cells expressing SERT at the time of analysis. For example, tdTomato will still be expressed at the time when cells that once expressed SERT are longer expressing it. Instead, the developmental timeline of SERT expression should be done with immunolabelling for SERT (such as Fig 1b). In addition, those immunolabellings should be displayed at lower magnification (as in

Feb.1b left image). One would need to know the fraction of tdtomato cells that are co-labelled with SERT. Related to the rest of the manuscript, it would be important to know whether the development of SERT is similar in males and females. Still, I do not see the point of the hub and ctip2 labellings as CA1 and CA3 can be more easily identified using anatomical criteria. One can observe a strong td Tomato labeling in the lacunosum molecular (1b left), which is probably related to the expression of the reporter in axonal tracts impinging onto this layer. This could indicate significant SERT expression in extra-hippocampal long-range inputs, the two more likely being ECL3 or the Ventro Medial thalamus. The authors may need to check this as it may complicate the interpretation of their LOF findings.

Responses: We have carried out quantitative analyses of the anatomical distribution of CA3 SERT-expressing neurons in each of the CA3a, b, c subfields in the ventral and dorsal hippocampus in 4 developmental stages in both males and females (Fig. 1D). Previous studies have established that the timing of the onset and developmental patterning of Cre-dependent reporter expression in SERTCre/+ mice may recapitulate the patterns of SERT mRNA in situ hybridization in those glutamatergic neurons as well as in the raphe serotonergic neurons (Narboux-Neme et al., Neuropharmacology, 2008). Cre-dependent GFP in the same SERTCre/+ mouse line has also been utilized as a reporter for illustrating the anatomical distribution and cell identity of SERT-expressing pyramidal neurons in the mPFC (Soiza-Reilly et al., Mol. Psychiatry, 2019). Following the reviewer' suggestion, we further assessed the percentage of SERT immuno-labeling of tdTomato reporter-expressing neurons in SERTCre/+ mice at 4 developmental stages – both quantitative data and images at a lower magnification are presented in Fig. S2.

We agree that CA3 and CA1 regions can be readily identified using anatomical criteria. However, Hub and Ctip2 immunostaining are important for confirming the CA3 cell identity of the SERT-expressing neurons. In particular, a few tdTom+ cells are located at the border between CA1, CA2 and CA3; Hub and Ctip2 immunostaining helps the identification of the CA3 cell identity. We have now moved the data including images and quantification of Hub and Ctip2 labeled tdTom-expressing neurons (previous Fig 1F) to Fig. S3.

Studies from multiple labs including ourselves found that SERT is not expressed in ECL3 but is strongly expressed in the thalamus (for an example, please see Fig. S1). To circumvent this caveat, we developed SERTPyramidΔ mice, which preserve SERT expression in the thalamic neurons as well as in the raphe serotonergic neurons (please see Fig. 2A), allowing us to specifically study the role of SERT expression in CA3 and mPFC pyramidal neurons.

Related to figure 3: The authors examine the impact of SERT LOF in CA3 pyramids on CA1 principal cells using a Thy1-GFP mouse line as in Deguchi et al. 2011. This raises several concerns. First, it is surprising that the authors do not investigate the direct effects of SERT LOF on CA3 neurons themselves (anatomy, excitability, connectivity, etc) but instead jump one synapse away from the initial insult. Also, the mouse line used to analyzed CA1 pyramids should be specified. Indeed, Deguchi et al use two different Thy1-GFP mouse lines which both label e subpopulation of CA1 cells, that might represent the earliest principal neurons in the hippocampus. The possible specificity of the subpopulation analyzed here should be taken into account. The authors need to consider the possibility that they may be analyzing the effects of SERT LOF on a subset of cells with specific trajectory. According to Deguchi et al, these cells should preferentially connect to CA3 neurons with a similar developmental time line: are the CA3 cells expressing SERT located in the early born subregions of CA3 (i.e. CA3a and b)?

Responses: As shown in Fig. 1E, SERT-expressing CA3 axons project along the stratum oriens and stratum radiatum to CA3 and CA1 at the ipsi-hippocampus and via the commissural pathway to innervate the contralateral hippocampus. Consequently, SERTPyramidΔ effects on CA3 pyramidal neurons may reflect a mix of ablating cell-autonomous CA3 SERT function and/or as the target of disrupting contralateral CA3 SERT function. Since CA1 pyramidal neurons are known postsynaptic partners of CA3 Schaffer collaterals, and LTP and LTD at CA3 Schaffer collaterals to CA1 synapses are the primary experimental models for investigating hippocampal circuit plasticity in vertebrates, we feel CA1 pyramidal neurons represent a good model for examining the effects of SERT-expressing CA3 pyramidal neurons at a target region. We evaluated basal SERTPyramidΔ CA3 neuron synaptic function with paired-pulse stimulus response profiles using pairs of stimuli at 10-500 msec

intervals – we observed no significant alteration in the CA3 Schaffer collateral presynaptic release probability at any interval tested (please see Fig 4A).

The reviewer is correct that different transgenic reporter lines driven by modified Thy1 promoter cassettes are expressed in different neuronal populations with shared distinct neurogenesis and synaptogenesis time window. We have now specified in the text (in addition to the Materials & Methods) that our Thy1-GFP mouse line is one of 25 alphabetically ordered Thy1 transgenic lines (i.e. Thy1-GFP line M or Thy1-GFP/M) described in Table 1 in Feng et al. Neuron, 2000 and is expressed in subpopulations of postnatally developing pyramidal neurons and DG granule cells -- not the Lsi1 and Lsi2 thy1-GFP/M reporter lines (where M stands for membrane-bound GFP reporter) analyzed in Deguchi et al. Nat. Neurosci. 2011. As the reviewer pointed out, Thy1-GFP-expressing CA1 pyramidal neurons may be preferentially connected to CA3 pyramidal neurons with similar developmental lines and our study may reflect SERT effects on a subset of cells with specific trajectory. We in fact examined several Thy1-GFP lines to select this line because it is selectively expressed in postnatally developing CA1 pyramidal neurons, matching to the period of CA3 SERT expression.

The number of Thy1-GFP cells is not different between control and SERT pyramids but the number of cells seems to vary a lot between age groups (eg. Around 1500 at P16 compared to 400 at P6), how can the authors explain this? Have the cells been sampled in the same location along the dorso-ventral and proximal-distal axes?

Response: As demonstrated in Fig. 3A and Fig. S5, this Thy1-GFP reporter line is selectively expressed in subpopulations of postnatally developing neurons. We quantified the number of GFP+ neurons in the pyramidal layer of the entire CA1 region in a defined number of serial sections starting from the hippocampal commissure and covering both dorsal and ventral hippocampus in the both hemispheres (Please see Materials and Methods for the details). Altman and Bayer established in mouse hippocampus that a significant portion of prenatally born pyramidal neuron precursors take developmental sojourn, attain the pyramidal layer postnatally and grow axons/dendrites during functional circuit assembly (Altman and Bayer, J. Comp Neurol, 1990). Evidence is growing that this late neuronal expansion is critical in shaping cognitive capability and complex behaviors (Silbereis et al., 2016; Stiles and Jernigan, 2010). We utilized this Thy1-GFP reporter as a model to test our hypothesis that CA3 SERT may influence hippocampal neural circuit establishment, in part, by regulating synaptic development of those late-developing CA1 pyramidal neurons.

Related to Fig 4. The authors show a very strong sex-dependent effect of SERT LOF on LTP/LTD with a blockage of LTD in both sexes and a boost of LTP in females. How can this major finding be linked to the previous analyses? How about LTP/LTD in juvenile mice that display less thin spines? Is expression of SERT and its localization the same in males and females?

Response: Despite the pronounced sex-biased prevalence and phenotype presentations in autism and related neurodevelopmental disorders, studies of disease-associated gene function in mouse models have focused on males. However, as explained in the Discussion, multiple mouse autism models (e.g. Tsc2+/- mice) display increased CA1 pyramidal neuron dendritic spine density and diminished LTD at the CA3-to-CA1 synapses, analogous to the phenotypes we have observed in SERTPyramidΔ mice. Remarkably, Tsc2+/- mice and another ASD mouse model, Fmr1 ko, both display increased CA1 pyramidal neuron dendritic spine density but show opposite changes in CA3-CA1 synaptic plasticity, with LTD diminished in Tsc2+/- mice while LTD enhanced and LTP diminished in Fmr1 ko mice (Piochon et al. Nature Neurosci, 2016; Penzes et al. Nat. Neurosci, 2011). Interestingly, Tsc2+/- mice and Fmr1 ko mice display overlapping behavioral deficits. In addition, increased dendritic spine density in postmortem brain of autism subjects and in Frm1 ko mice are developmental stage dependent. These observations highlight complexed relationships between anatomical and functional alterations in the hippocampus. Our work extends previous studies by demonstrating that SERT expression in the pyramidal neurons during development is essential for achieving normal CA3-CA1 synaptic plasticity in adulthood. Following the reviewer's question, we have further quantified anatomical localization of SERT-expressing CA3 neurons in males and females, and found no significant differences between the two sexes (Fig. 1D). A prevailing view in the field is that an overt trait may be similar but underlying mechanisms and therefore functional impacts may distinctly differ. We have clearly acknowledged in the text that

future studies are required to elucidate how disruption of developmental SERT function may lead to aberrant LTP/LTD at the adult hippocampus in males versus females.

Related to fig. 6: the authors observe subtle changes in expression levels of genes in SERT LOF experiments at P7. They choose to analyze the datasets with p<0.05, which represents the lowest significance level. Why not focus on the smallest list of DEGs obtained at p<0.005? Would the conclusions be different? Why choose P7 since it corresponding to the peak of SERT expression? Maybe adult or juvenile analysis would have made more sense if one wants to relate this to the observed juvenile/adult modifications in CA1 anatomy and plasticity.

Response: We thank the reviewer for the thoughtful comments and excellent suggestion. Indeed, changes in the DEGs in the SERTPyramidΔ hippocampus are subtle in magnitude. Subtle changes in gene expression levels have been observed in transcriptomes of the postmortem brain of autism and schizophrenia. We performed GO analyses with DEGs at P<0.05, to test the idea that SERT function may fine tune multiple functionally-related components in certain cellular and biological processes, rather than being essential for the expression of a small set of genes. Following reviewer's suggestion, we have further analyzed the datasets by performing GO analyses with the DEGs at P<0.01 and P<0.005. We identified that DEGs at P<0.01 and P<0.005 remained sex specific, displayed similar patterns and were functionally related but in fewer GO terms identified with DEGs at P<0.05; data are presented in Tables S4-S8 and discussed in the text.

In contrast to nearly all disease-associated genes studied to date that are continuously expressed in the brain, SERT is specifically expressed in specific subsets of pyramidal neurons and only in a narrow developmental window. We feel that the transcriptome of P7 hippocampus at the peak of CA3 SERT expression may present a unique opportunity to identify the developmental origin of gene expression patterns that might be critical for normal hippocampal functional circuit maturation and are impaired by reduced SERT function, either by SERT gene variants or by early life SSRI exposures, as well as by diverse disease-associated genetic variants.

Second decision letter

MS ID#: DEVELOP/2022/200549

MS TITLE: Sex-biased effects on hippocampal circuit development by perinatal SERT expression in CA3 pyramidal neurons

AUTHORS: Roberto De Gregorio, Galadu Subah, Jennifer Chan, Luisa Speranza, Xiaolei Zhang, Aarthi Ramakrishnan, Li Shen, Ian Maze, Patric K Stanton, and Jiying Y Sze

I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that you address the remaining minor comments of one of the referees. Please attend to all of the reviewer' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 3

Advance summary and potential significance to field

This manuscript by De Gregorio and colleagues shows that the serotonine uptake transporter (SERT) is transiently expressed in CA3 cells during development until the end of the first postnatal week and that a disruption of SERT expression in these cells leads to sex-biased defects at genetic,

functional and behavioral levels. This set of observations is novel, intriguing and potentially important.
It covers several aspects of hippocampal function and span many scales of analysis, from genes to circuits and behavior.

Comments for the author

The authors have addressed most of my concerns. I still think that the scope of the analysis is too broad and that datasets could have been better connected, but nevertheless recommend publication. I only have a few remaining remarks (see re-responses).

1) The basic early steps of circuit dysfunction during development are overlooked, including the fate of the CA3 neurons and their wiring to their postsynaptic CA1 targets.
Response: This is the first characterization of the biological roles of SERT expression in the pyramidal neurons for hippocampal development. We feel that the systematic genetic, anatomical, circuit and behavioral analyses are necessary to establish that the transient SERT expression during this developmental stage is essential for achieving normal hippocampal synaptic circuitry in adulthood and behavior. Following reviewer's question, we have confirmed that SERT is not required for general cell fate specification of the CA3 SERT-expressing neurons (Fig. S3D).

Re-response: This was not really my point, sorry for the misunderstanding. By "fate of CA3 neurons" I did not mean "specification"; instead, I was alluding to the development of their morpho-physiological features in the juvenile brain. But I guess this was not done here and remains for future work.

2) Related to figure 1comment:
Responses: We have carried out quantitative analyses of the anatomical distribution of CA3 SERT-expressing neurons in each of the CA3a, b, c subfields in the ventral and dorsal hippocampus in 4 developmental stages in both males and females (Fig. 1D). Previous studies have established that the timing of the onset and developmental patterning of Cre-dependent reporter expression in SERTCre/+ mice may recapitulate the patterns of SERT mRNA in situ hybridization in those glutamatergic neurons as well as in the raphe serotonergic neurons (Narboux-Neme et al., Neuropharmacology, 2008).
Cre-dependent GFP in the same SERTCre/+ mouse line has also been utilized as a reporter for illustrating the anatomical distribution and cell identity of SERT-expressing pyramidal neurons in the mPFC (Soiza-Reilly et al., Mol. Psychiatry 2019).

Re-response: It is well known that Cre-dependent reporter expression depends on the reporter, so my suggestion was fully justified and the authors have now addressed it in Fig. S2.

3) Related to figure 3:
The number of Thy1-GFP cells is not different between control and SERT pyramids but the number of cells seems to vary a lot between age groups (eg. Around 1500 at P16 compared to 400 at P6), how can the authors explain this? Have the cells been sampled in the same location along the dorso-ventral and proximal-distal axes?
Response: As demonstrated in Fig. 3A and Fig. S5, this Thy1-GFP reporter line is selectively expressed in subpopulations of postnatally developing neurons. We quantified the number of GFP+ neurons in the pyramidal layer of the entire CA1 region in a defined number of serial sections starting from the hippocampal commissure and covering both dorsal and ventral hippocampus in the both hemispheres (Please see Materials and Methods for the details). Altman and Bayer established in mouse hippocampus that a significant portion of prenatally born pyramidal neuron precursors take developmental sojourn, attain the pyramidal layer postnatally and grow axons/dendrites during functional circuit assembly (Altman and Bayer, J. Comp Neurol, 1990). Evidence is growing that this late neuronal expansion is critical in shaping cognitive capability and complex behaviors (Silbereis et al., 2016; Stiles and Jernigan, 2010). We utilized this Thy1-GFP reporter as a model to test our hypothesis that CA3 SERT may influence hippocampal neural circuit establishment, in part, by regulating synaptic development of those late-developing CA1 pyramidal neurons.

Re-response: This does not fully address my comment about the different numbers of Thy1-GFP cells at P6 and P16. By P7 all CA1 neurons have migrated into CA1.

4) Related to Fig 4. The authors show a very strong sex-dependent effect of SERT LOF on LTP/LTD with a blockage of LTD in both sexes and a boost of LTP in females. How can this major finding be linked to the previous analyses? How about LTP/LTD in juvenile mice that display less thin spines? Is expression of SERT and its localization the same in males and females?
Response: Despite the pronounced sex-biased prevalence and phenotype presentations in autism and related neurodevelopmental disorders, studies of disease-associated gene function in mouse models have focused on males. However, as explained in the Discussion, multiple mouse autism models (e.g. Tsc2+/- mice) display increased CA1 pyramidal neuron dendritic spine density and diminished LTD at the CA3-to-CA1 synapses, analogous to the phenotypes we have observed in SERTPyramidΔ mice.
Remarkably, Tsc2+/- mice and another ASD mouse model, Fmr1 ko, both display increased CA1 pyramidal neuron dendritic spine density but show opposite changes in CA3-CA1 synaptic plasticity, with LTD diminished in Tsc2+/- mice while LTD enhanced and LTP diminished in Fmr1 ko mice (Piochon et al. Nature Neurosci, 2016; Penzes et al. Nat. Neurosci, 2011). Interestingly, Tsc2+/- mice and Fmr1 ko mice display overlapping behavioral deficits. In addition, increased dendritic spine density in postmortem brain of autism subjects and in Frm1 ko mice are developmental stage dependent. These observations highlight complexed relationships between anatomical and functional alterations in the hippocampus.

Re-response: Ok, but this does not directly address my comment about LTP/LTD in juvenile mice that display les spines. I guess this remains to be done in future investigations.

Second revision

Author response to reviewers' comments

De Gregorio et al. DEVELOP/2022/200549
We thank the editor and the three reviewers for the positive evaluation of our revised manuscript. In this resubmission, we further address Reviewer #3's comments. Please see our point-to-point response below.

Reviewer 3 Advance summary and potential significance to field This manuscript by De Gregorio and colleagues shows that the serotonine uptake transporter (SERT) is transiently expressed in CA3 cells during development until the end of the first postnatal week and that a disruption of SERT expression in these cells leads to sex-biased defects at genetic, functional and behavioral levels. This set of observations is novel, intriguing and potentially important. It covers several aspects of hippocampal function and span many scales of analysis, from genes to circuits and behavior.

Responses: We thank the reviewer for the evaluation of our work.

Reviewer 3 Comments for the author The authors have addressed most of my concerns. I still think that the scope of the analysis is too broad and that datasets could have been better connected, but nevertheless recommend publication. I only have a few remaining remarks (see re-responses).

1) The basic early steps of circuit dysfunction during development are overlooked, including the fate of the CA3 neurons and their wiring to their postsynaptic CA1 targets.

Response: This is the first characterization of the biological roles of SERT expression in the pyramidal neurons for hippocampal development. We feel that the systematic genetic, anatomical, circuit and behavioral analyses are necessary to establish that the transient SERT expression during this developmental stage is essential for achieving normal hippocampal synaptic circuitry in adulthood and behavior. Following reviewer's question, we have confirmed that SERT is not required for general cell fate specification of the CA3 SERT-expressing neurons (Fig. S3D).

Re-response: This was not really my point, sorry for the misunderstanding. By "fate of CA3 neurons" I did not mean "specification"; instead, I was alluding to the development of their

morpho-physiological features in the juvenile brain. But I guess this was not done here and remains for future work.

Response to Re-response: Yes, future studies will be designed to elucidate the effects of the transient SERT expression in the CA3 pyramidal neurons on the development of aspects of hippocampal synaptic circuits in males vs. females and the underlying molecular mechanisms.

However, in addition to the verification that SERT is not required for CA3 pyramidal neuron cell fate specification (Fig S3D), the current study examined SERTPyramidΔ effects on several aspects of CA3 and CA1 synaptic development and function. a) We examined CA1 pyramidal neuron dendritic structure at P16, a timing when the CA3-CA1 functional circuits are just established (please see Fig. 3). We performed 3D-reconstruction analyses of the entire CA1 pyramidal neuron apical dendritic tree and 3rd order dendritic branches located at the stratum radiatum – the CA3 Schaffer collateral innervation field (Fig. 3C). We further performed morphometric analyses of spines of the 3rd order dendritic branches (Fig. 3E, F). We observed that CA3 SERT expression is essential for normal development of CA1 pyramidal neuron dendritic spine, but is not required for the general dendrite growth.
b) We examined several aspects of CA3-CA1 synaptic activity in SERTPyramidΔ mice vs. control littermates age 7 – 8 weeks old, using electrophysiology (please see Fig. 4). We evaluated basal CA3 pyramidal neuron synaptic function with paired-pulse stimulus response profiles using pairs of stimuli at 10-500 msec intervals – we observed no significant differences in CA3 Schaffer collateral presynaptic release probability at any interval tested between the two genotype groups (Fig 4A). In contrast, we observed sex-biased deficits in long-term activity-dependent synaptic plasticity of CA3-CA1 synapses (Fig. 4B, C). These observations together suggest that the general CA3 pyramidal neuron characteristics such as their projection to CA1 and the basic presynaptic release ability at CA3-CA1 synapses are preserved in SERTPyramidΔ mice, and that observed changes in the long-term synaptic plasticity involve, at least in part, altered activity-dependent mechanisms of the CA1 pyramidal neurons.

Our future studies will investigate into the molecular and cellular mechanisms underlying the changes in CA1 pyramidal neuron spine density and CA3-CA1 synaptic plasticity in SERTPyramidΔ mice.

2)Related to figure 1comment:
Responses: We have carried out quantitative analyses of the anatomical distribution of CA3 SERT-expressing neurons in each of the CA3a, b, c subfields in the ventral and dorsal hippocampus in 4 developmental stages in both males and females (Fig. 1D). Previous studies have established that the timing of the onset and developmental patterning of Cre-dependent reporter expression in SERTCre/+ mice may recapitulate the patterns of SERT mRNA in situ hybridization in those glutamatergic neurons as well as in the raphe serotonergic neurons (Narboux-Neme et al., Neuropharmacology, 2008). Cre-dependent GFP in the same SERTCre/+ mouse line has also been utilized as a reporter for illustrating the anatomical distribution and cell identity of SERT-expressing pyramidal neurons in the mPFC (Soiza-Reilly et al., Mol. Psychiatry, 2019).

Re-response: It is well known that Cre-dependent reporter expression depends on the reporter, so my suggestion was fully justified and the authors have now addressed it in Fig. S2.

Response to Re-response: We thank the reviewer for the important suggestion and the appreciation of our revision work.

3)Related to figure 3:

The number of Thy1-GFP cells is not different between control and SERT pyramids but the number of cells seems to vary a lot between age groups (eg. Around 1500 at P16 compared to 400 at P6), how can the authors explain this? Have the cells been sampled in the same location along the dorso-ventral and proximal-distal axes?

Response: As demonstrated in Fig. 3A and Fig. S5, this Thy1-GFP reporter line is selectively expressed in subpopulations of postnatally developing neurons. We quantified the number of GFP+

neurons in the pyramidal layer of the entire CA1 region in a defined number of serial sections starting from the hippocampal commissure and covering both dorsal and ventral hippocampus in the both hemispheres (Please see Materials and Methods for the details). Altman and Bayer established in mouse hippocampus that a significant portion of prenatally born pyramidal neuron precursors take developmental sojourn, attain the pyramidal layer postnatally and grow axons/dendrites during functional circuit assembly (Altman and Bayer, J. Comp Neurol, 1990). Evidence is growing that this late neuronal expansion is critical in shaping cognitive capability and complex behaviors (Silbereis et al., 2016; Stiles and Jernigan, 2010). We utilized this Thy1-GFP reporter as a model to test our hypothesis that CA3 SERT may influence hippocampal neural circuit establishment, in part, by regulating synaptic development of those late-developing CA1 pyramidal neurons.

Re-response: This does not fully address my comment about the different numbers of Thy1-GFP cells at P6 and P16. By P7 all CA1 neurons have migrated into CA1.

Response to Re-response: As the reviewer pointed out in the initial review, different transgenic lines driven by modified Thy1 promoter cassettes are expressed in different neuronal populations with shared distinct neurogenesis and synaptogenesis time window. The goal of this study was to utilize the published Thy1-GFP/M transgenic mouse line as a reporter enabling us to characterize the same population of CA1 pyramidal neurons in SERTPyramidΔ mice vs. control littermates, to reduce the variations due to different neuronal subtypes. We observed that Thy1-GFP/M-expressing neurons emerged at the pyramidal layer and the dentate gyrus (DG) at P3, and the number of the GFP+ neurons progressively increased to reach the adult level around P16. BrdU birth dating showed that the Thy1-GFP/M-expressing CA1 pyramidal neurons and DG neurons were generated around a peak of E14.5 and E18.5, respectively (Fig. S5A). However, the GFP+ neurons displayed immature characteristics even at P6, judged by ~12% and 25% of the GFP+ CA1 pyramidal neurons and DG neurons expressing the immature neuronal marker Sox2 and > 80% of the GFP+ CA1 pyramidal neurons lacking 3rd order apical dendritic branches. In contrast, nearly all the GFP+ CA1 pyramidal neurons displayed mature neuronal characteristics at P16 (please see Fig. S5B). These data suggest that Thy1-GFP/M is selectively expressed in a neuronal subpopulation that undergo developmental sojourn and then complete the terminal differentiation postnatally as described by J. Altman and S.A Bayer (J. of Comp. Neuro.1990). We observed no significant differences in the number of Thy1-GFP/M-expressing CA1 pyramidal neurons at P3, P6 and P16 and the general dendrite growth between SERTPyramidΔ mice and littermate controls (please see Fig. 3B, C). In marked contrast, we observed significantly reduced density of thin spines on the 3rd order dendrites of the GFP+ CA1 pyramidal neurons in P16 SERTPyramidΔ mice (Fig. 3E, F). These observations indicate that SERT function regulates dendritic spine development of this population of CA1 pyramidal neurons but is not required for their ability to attain CA1 or the general dendrite growth.

We previously addressed the reviewer's question "have the cells been sampled in the same location along the dorso-ventral and proximo-distal axes?" As detailed in the Methods, we quantified the number of the GFP+ neuron in the pyramidal layer of the entire CA1 region in a defined number of serial sections starting from the hippocampal commissure covering both dorsal and ventral hippocampus in both hemispheres. The mechanism underlying the increased number of Thy1-GFP-expressing CA1 pyramidal neurons at P16 vs. P6 is not known. 85% of hippocampal neurons in rodents attain the terminal destination during the first three postnatal weeks (Bayer SA, J. Comp Neurol. 1980; Bandeira et al. PNAS, 2009). The reviewer is correct that all or at least the vast majority of CA1 pyramidal neurons have migrated to CA1 by P7. One speculation is that Thy1-GFP/M-expressing neurons represent a small neuronal population attaining CA1 during the second postnatal week that may not be discernable with previous methods assessing general CA1 neuronal populations. Since Thy1-GFP/M-expressing CA1 pyramidal neurons mature during the first two postnatal weeks (Fig. S5B), another speculation is that these neurons indeed attain CA1 earlier but express the GFP later during the terminal differentiation. However, since SERT is not required for regulating the number of Thy1-GFP/M-expressing neurons in the CA1, we feel that determination of the mechanisms underlying the increased number of the GFP+ neurons in P16 vs. P6 is beyond the scope of this study.

4)Related to Fig 4. The authors show a very strong sex-dependent effect of SERT LOF on LTP/LTD with a blockage of LTD in both sexes and a boost of LTP in females. How can this major finding be linked to the previous analyses? How about LTP/LTD in juvenile mice that display less thin spines? Is expression of SERT and its localization the same in males and females?

Response: Despite the pronounced sex-biased prevalence and phenotype presentations in autism and related neurodevelopmental disorders, studies of disease-associated gene function in mouse models have focused on males. However, as explained in the Discussion, multiple mouse autism models (e.g. Tsc2+/- mice) display increased CA1 pyramidal neuron dendritic spine density and diminished LTD at the CA3-to-CA1 synapses, analogous to the phenotypes we have observed in SERTPyramidΔ mice. Remarkably, Tsc2+/- mice and another ASD mouse model, Fmr1 ko, both display increased CA1 pyramidal neuron dendritic spine density but show opposite changes in CA3-CA1 synaptic plasticity, with LTD diminished in Tsc2+/- mice while LTD enhanced and LTP diminished in Fmr1 ko mice (Piochon et al. Nature Neurosci, 2016; Penzes et al. Nat. Neurosci, 2011). Interestingly, Tsc2+/- mice and Fmr1 ko mice display overlapping behavioral deficits. In addition, increased dendritic spine density in postmortem brain of autism subjects and in Frm1 ko mice are developmental stage dependent. These observations highlight complexed relationships between anatomical and functional alterations in the hippocampus.

Re-response: Ok, but this does not directly address my comment about LTP/LTD in juvenile mice that display les spines. I guess this remains to be done in future investigations.

Response to Re-response: Yes, we will build on the genetic, anatomical and LTP/LTD phenotypes described in this paper to further delineate SERTPyramidΔ effects on aspects of synaptic connectivity/plasticity and the molecular and cellular mechanisms regulated by SERT during hippocampal circuit establishment as well as long-lasting effects on the adult hippocampal synaptic circuitry.

Third decision letter

MS ID#: DEVELOP/2022/200549

MS TITLE: Sex-biased effects on hippocampal circuit development by perinatal SERT expression in CA3 pyramidal neurons

AUTHORS: Roberto De Gregorio, Galadu Subah, Jennifer Chan, Luisa Speranza, Xiaolei Zhang, Aarthi Ramakrishnan, Li Shen, Ian Maze, Patric K Stanton, and Jiying Y Sze
ARTICLE TYPE: Research Article

I am delighted to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.