Selective refinement of glutamate and GABA synapses on dorsal raphe 5-HT neurons during postnatal life

Carla V. Argañaraz, Tamara S. Adjimann, Paula P. Perissinotti and Mariano Soiza-Reilly
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MS TITLE: Selective refinement of glutamate and GABA synapses on dorsal raphe 5-HT neurons during postnatal life

AUTHORS: Carla V Arganaraz, Tamara S Adjimann, Paula P Perissinotti, and Mariano Soiza-Reilly

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.

As you will see, the referees express considerable interest in your work, but have some 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 receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' 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

The manuscript entitled "Selective refinement of glutamate and GABA synapses on dorsal raphe 5-HT neurons during postnatal life" examined the alterations of glutamate and GABA synapses in 5-HT

neurons during postnatal development with morphological and electrophysiological approaches, very impressed with the data from the high-resolution quantitative microscopy. They found that GABA and cortical glutamate synaptic inputs to DRN 5-HT neurons undergo a profound refinement process after the third postnatal week, while subcortical glutamate synapses do not. These results are important for understanding of neural maladaptations involved in the neurodevelopmental vulnerability to 5-HT-associated psychiatric disorders.

Comments for the author

This reviewer has some suggestions/comments that maybe helpful to revise this manuscript.

1. Fig 1. High quality of morphological data, very impressed. There is one point that should be revised. That is, VGLUT1 is expressed in cortical axons while VGLUT2 is present in axonal projections arising from subcortical structures (Soiza-Reilly and Commons, 2011a; Soiza-Reilly and Commons, 2011b).
The vast majority of the axons does so but not all of them. Expressions related to this are “cortical glutamate input” and “subcortical glutamate input”. In addition, please rephrase “cortical glutamate and GABA synaptic inputs”, as it may lead to misunderstanding that GABA input is from the cortex.

2. Fig. 2. The data collected are sufficient to support the claim, well done.

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Fig. 4. The data are meaningful and supportive, but the statement is somehow overstated. If you want to say “Lack of re-scaling of 5-HT1A receptor-mediated inhibition on 5-HT neurons during synaptic refinement”, more data are absolutely needed. It (this section) should be revised, also related statements throughout the manuscript (e.g., Abstract, Introductions), based on the data obtained. In addition, I guess the c-Fos+ cells in Y axis (Fig. 4D) mean 5-HT neurons with c-Fos (double labeled), please clarify this, also add related information in Materials and Methods, such as how the cells were counted.

Reviewer 2

Advance summary and potential significance to field

This is a very interesting study in line with previous excellent work from Soiza-Reilly on the development of cortical-to-dorsal raphe nucleus (DRN) 5-HT neurons circuits. The manuscript by Arganaraz and collaborators reports on the use of high-resolution quantitative microscopy technique array tomography combined with ex-vivo electrophysiological recordings to investigate the postnatal trajectory of glutamate and GABA synaptic inputs to dorsal raphe 5-HT neurons. Results identify a time window between the third and fourth postnatal week in which a profound synaptic refinement of DRN excitatory and inhibitory afferents is observed.

Comments for the author

The manuscript in its present form has some flaws that need to be revised by the authors.

I do have few concerns that need to be dispelled with some of the suggested experiments or, alternatively, that should be appropriately discussed.

A) The paragraph (Line 138-143) requires a rewrite, since the language used is rather ambiguous. The authors make the claim that “changes in the size of the axon boutons could result in apparent variations in synaptic densities due to fluctuations in fluorescence intensity”. Does this mean that the increase in fluorescence observed in the dataset is linked with an increased synaptic density that can be accounted for by the increase in bouton volume? Does it mean that with the increase in the volume of axonal boutons would lead to a pre or postsynaptic apparent variations in the number or size of the synaptic density which will lead to a bias in counting the synaptic markers?

Since the previous experiments only dealt with counting the synaptic markers and comparing between different time points, it seems arbitrary that this should be mentioned here. To accurately test whether an increase in synaptic density or volume of boutons or dendritic spines is present, more suitable approach would better tackle this questions, e.g. e.m. study. The authors resolve was to measure the size of synaptic boutons, however it is not specifically stated why an analysis of bouton size or volume will shed more light on this subject. Does the fact that all boutons increase their size throughout postnatal life increase their synaptic density as well, leading to a more accurate reading of the data set? It is well-known that increases in bouton size is correlated with a larger synaptic density, which holds true also for postsynaptic densities (1, 2), but the relevance in this experiment must be more clearly stated. The second part of the paragraph seem to take the age-related enlargement of axonal bouton as a direct link which gives even more data towards supporting a process of synaptic pruning in the DRN. However, this process of bouton enlargement can be attributed to long term potentiation (LTP) mechanisms based on the individual activity pattern of neurons. In this way, active neurons that escaped the process of neuronal pruning due to their enhanced activity pattern acquired different morphological features which allowed for an increase in size. However, this process is in opposition to synaptic pruning, in which synapses, dendrites, axons and neurons are phagocytized by microglia. Recent studies (3) also showed that biochemical pathways used in the induction of Long term depression (LTD-which is the counterpart of LTP, leading to opposite effects on neuronal activity) are similar to the ones used in synaptic pruning, making the LTD process a better biomarker for synaptic pruning rather than LTP. Thus the inclusion of this paragraph in the manuscript must be addressed more carefully or removed.

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3. Piochon C, Kano M, Hansel C. LTD-like molecular pathways in developmental synaptic pruning. Nat Neurosci. 2016 Sep 27;19(10):1299-310. doi: 10.1038/nn.4389. PMID: 27669991; PMCID: PMC5070480.

B) The paragraph (180-204) states that the morphological differences observed with the array tomography analysis can be replicated with electrophysiology data. However, this only holds true for GABAergic synapses in which a reduction in GAD2 puncta was correlated with a decrease in firing frequency. The authors then separated glutamatergic input onto 5-HT neurons in cortical vs. sub-cortical but no electrophysiology experiments were conducted in this paradigm. It would have been of great interest to observe electrophysiological changes in cortical afferents, where a decrease in VGLUT1+ puncta was observed, as with the case of GABAergic input, and no differences in sub-cortical afferents to 5-HT neurons in VGLUT2+ axon terminals that were shown to not undergo a decrease with the array tomography analysis. Also, the figure 3 seems to just replicate some of the findings from figure 1, with no apparent contribution to the story. Thus it is unclear whether the lack of differences observed in the electrophysiological experiments for the glutamatergic inputs are to the fact that VGLUT2+ sub-cortical afferents can develop stronger synaptic contacts (LTP) that can, in time, act as a balancing mechanism which can counteract the synaptic pruning underwent by the VGLUT1+ terminals. But for this, more in depth electrophysiological experiments must be performed. I would recommend that these experiments need to be performed before an accurate analysis the functional aspects of synaptic pruning can be established.

C) Paragraphs (215-229) are aimed at reconciling the observed pruning of cortical glutamatergic input to 5-HT neurons that occur during this critical period. The array tomography analysis does indeed show a difference in synaptic pruning from the two separate glutamatergic systems. However, it draws short of explaining the functional modifications that can arise from such a pruning. For example, during the critical period observed here, does the VGLUT2+ afferents show a higher firing frequency that could account for no differences in the overall firing of 5-HT neurons? Are these two mechanisms working in a synergistic manner through which process of pruning is more directed to synapses that receive only VGLUT 1 as opposed to both VGLUT1 and VGLUT2?

Some of these questions can really shed a positive light on the development of the Raphe-PFC pathway and how it is altered in disease. Thus, an experimental paradigm that would perform the same electrophysiological measurements in both these subpopulations coupled with a loss of function manipulation during animal development would bring the outcome of the research to a more conclusive story. Another area of questioning that can be resolved by such experiments is whether there is an intrinsic need for a much more pronounced glutamatergic input to 5-HT neurons from sub-cortical areas during post natal life than from cortical areas, and whether this synergistic approach to synaptic pruning is necessary to achieve the excitatory/inhibitory synaptic drives upon DRN neurons and how alteration of this pruning mechanism is involved in maladaptive mechanisms increasing the risk of developing mental disorders later in life.

First revision

Author response to reviewers' comments

Response to Reviewer #1:

The manuscript entitled "Selective refinement of glutamate and GABA synapses on dorsal raphe 5-HT neurons during postnatal life" examined the alterations of glutamate and GABA synapses in 5-HT neurons during postnatal development with morphological and electrophysiological approaches, very impressed with the data from the high-resolution quantitative microscopy. They found that GABA and cortical glutamate synaptic inputs to DRN 5-HT neurons undergo a profound refinement process after the third postnatal week, while subcortical glutamate synapses do not. These results are important for understanding of neural maladaptations involved in the neurodevelopmental vulnerability to 5-HT-associated psychiatric disorders.

We thank the reviewer for their comments.

This reviewer has some suggestions/comments that maybe helpful to revise this manuscript.

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We thank the reviewer for their comment. We have now clarified this point and rephrased it throughout the manuscript.

2) Fig. 2. The data collected are sufficient to support the claim, well done.

We thank the reviewer for their comment.

3) Fig. 3. Tph2 staining is a reliable tool to trace dendrites of 5-HT neurons, again data are shown with high quality.

We thank the reviewer for their comment.

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We thank the reviewer for their comment. We have now clarified these points and edited the manuscript and Figure 4 accordingly (highlighted in blue).

Response to Reviewer #2:

This is a very interesting study in line with previous excellent work from Soiza-Reilly on the development of cortical-to-dorsal raphe nucleus (DRN) 5-HT neurons circuits. The manuscript by Argañaraz and collaborators reports on the use of high-resolution quantitative microscopy technique array tomography combined with ex-vivo electrophysiological recordings to investigate the postnatal trajectory of glutamate and GABA synaptic inputs to dorsal raphe 5-HT neurons. Results identify a time window between the third and fourth postnatal week in which a profound synaptic refinement of DRN excitatory and inhibitory afferents is observed.

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I do have few concerns that need to be dispelled with some of the suggested experiments or, alternatively, that should be appropriately discussed.

A) The paragraph (Line 138-143) requires a rewrite, since the language used is rather ambiguous. The authors make the claim that "changes in the size of the axon boutons could result in apparent variations in synaptic densities due to fluctuations in fluorescence intensity". Does this mean that the increase in fluorescence observed in the dataset is linked with an increased synaptic density that can be accounted for by the increase in bouton volume? Does it mean that with the increase in the volume of axonal boutons would lead to a pre or postsynaptic apparent variations in the number or size of the synaptic density which will lead to a bias in counting the synaptic markers? Since the previous experiments only dealt with counting the synaptic markers and comparing between different time points, it seems arbitrary that this should be mentioned here.

We apologize for the confusion and are very grateful to the reviewer for pointing this out. We have removed the mentioned paragraph and edited this section substantially. We want to make clear that the number of synaptic boutons quantified in our study is not affected by changes in the volume of detected objects. Unless, of course, the bouton's size is overly reduced and its signal falls under the fluorescence detection limits. The extremely high sensitivity of array tomography would allow to detect these instances and quantify them with a very high accuracy. Interestingly, we found that the size of synaptic boutons increases with age, further supporting a process of synaptic pruning in the DRN. This point has been clarified in the manuscript (highlighted in blue).

To accurately test whether an increase in synaptic density or volume of boutons or dendritic spines is present, more suitable approach would better tackle this questions, e.g. e.m. study.

We thank the reviewer for the suggestion. However, no evident ultraestructural differences were previously reported when comparing VGLUT1+ and VGLUT2+ synapses in the adult DRN (Commons et al., 2005). We now added this reference to the manuscript (highlighted in blue).

The authors resolve was to measure the size of synaptic boutons, however it is not specifically stated why an analysis of bouton size or volume will shed more light on this subject. Does the fact that all boutons increase their size throughout postnatal life increase their synaptic density as well, leading to a more accurate reading of the data set? It is well-known that increases in bouton size is correlated with a larger synaptic density, which holds true also for postsynaptic densities (1, 2), but the relevance in this experiment must be more clearly stated.

We agree with the reviewer on the relevance of how the size of the synaptic bouton could impact postsynaptic connectivity, however we think that this topic falls beyond the scope of the present study.
The second part of the paragraph seem to take the age-related enlargement of axonal bouton as a direct link which gives even more data towards supporting a process of synaptic pruning in the DRN. However, this process of bouton enlargement can be attributed to long term potentiation

(LTP) mechanisms based on the individual activity pattern of neurons. In this way, active neurons that escaped the process of neuronal pruning due to their enhanced activity pattern acquired different morphological features which allowed for an increase in size. However, this process is in opposition to synaptic pruning, in which synapses, dendrites, axons and neurons are phagocytized by microglia. Recent studies (3) also showed that biochemical pathways used in the induction of Long term depression (LTD-which is the counterpart of LTP, leading to opposite effects on neuronal activity) are similar to the ones used in synaptic pruning, making the LTD process a better biomarker for synaptic pruning rather than LTP. Thus the inclusion of this paragraph in the manuscript must be addressed more carefully or removed.

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We thank the reviewer for the insightful comment. We have now included a paragraph in the discussion related to pruning, microglial activity and LTD, using one of the references mentioned by the reviewer. In addition, as we mentioned before, we have edited this section and removed the original confusing paragraph from the manuscript (highlighted in blue).

B) The paragraph (180-204) states that the morphological differences observed with the array tomography analysis can be replicated with electrophysiology data. However, this only holds true for GABAergic synapses in which a reduction in GAD2 puncta was correlated with a decrease in firing frequency. The authors then separated glutamatergic input onto 5-HT neurons in cortical vs. sub-cortical but no electrophysiology experiments were conducted in this paradigm. It would have been of great interest to observe electrophysiological changes in cortical afferents, where a decrease in VGLUT1+ puncta was observed, as with the case of GABAergic input, and no differences in sub-cortical afferents to 5-HT neurons in VGLUT2+ axon terminals that were shown to not undergo a decrease with the array tomography analysis.

The reviewer has a point. In our electrophysiological recordings glutamate synaptic currents are examined without identifying specific populations of synapses that may arise from different sources (i.e. VGLUT1+ vs. VGLUT2+ synaptic inputs). VGLUT1+ synapses preferentially associate with small-caliber and more distal 5-HT dendritic processes and spines, while VGLUT2+ synapses are often found in larger dendritic shafts closer to 5-HT somas (Commons et al., 2005; Soiza-Reilly and Commons, 2011a). In an attempt to functionally identify these two populations of glutamate inputs in our recordings at P21 and P28, we fitted the histograms of mEPSC half-width durations to two-peak Gaussian models (new Suppl. Fig. 2). This analysis allowed us to identify two populations of mEPSCs with different kinetics that could correspond to VGLUT1+ and VGLUT2+ synapses (new Suppl. Fig. 2). With this approach, we found that slower glutamate miniature events were more frequent at P21, while at P28 faster events become more predominant (new Suppl. Fig. 2). Consistent with this, mEPSCs displayed shorter decay times at P28 (Mann-Whitney U=11, p= 0.028 for P21 vs. P28) (new Fig. 3E). Kinetic differences detected may be reflecting specific age-related changes in VGLUT1+ synapses that are expected to produce slower miniatures in comparison to VGLUT2+ synapses that are located closer to 5-HT cell bodies (Commons et al., 2005; Soiza-Reilly and Commons, 2011a).

Now we have included these new data in the manuscript (new Suppl. Fig. 2), supporting the presence of two populations of mEPSCs onto DRN 5-HT neurons, likely corresponding to VGLUT1+ and VGLUT2+ synapses. These new electrophysiological results show that both populations undergo age-dependent modifications that are very consistent with our array tomography data.

Also, the figure 3 seems to just replicate some of the findings from figure 1, with no apparent contribution to the story. Thus it is unclear whether the lack of differences observed in the

electrophysiological experiments for the glutamatergic inputs are to the fact that VGLUT2+ sub-cortical afferents can develop stronger synaptic contacts (LTP) that can, in time, act as a balancing mechanism which can counteract the synaptic pruning underwent by the VGLUT1+ terminals. But for this, more in depth electrophysiological experiments must be performed. I would recommend that these experiments need to be performed before an accurate analysis the functional aspects of synaptic pruning can be established.

We thank the reviewer for their comment. We now clarified the relevance of Figure 3 in our study, and re-organized the Figures to optimize the flow of the manuscript to the readers. Now, in Figure 1 we show the global changes upon synaptic afferents in the DRN, while in new Figure 2 we analyzed specific synapses exclusively associated with 5-HT neurons. New Figure 3 shows electrophysiological data and Figure 4 c-Fos results. We agree it will be very interesting to explore the possibility that plasticity changes in VGLUT2+ synapses could be counteracting the synaptic refinement underwent by VGLUT1+ synapses. However, to test this possibility, a different electrophysiological setup is needed, incorporating the use of optogenetic tools during early-life to perform input-specific stimulations and plasticity protocols during postnatal development. We believe that although it would be very interesting, those investigations are beyond the scope of the present study. We now mentioned these two points in the discussion (highlighted in blue).

C) Paragraphs (215-229) are aimed at reconciling the observed pruning of cortical glutamatergic input to 5-HT neurons that occur during this critical period. The array tomography analysis does indeed show a difference in synaptic pruning from the two separate glutamatergic systems. However, it draws short of explaining the functional modifications that can arise from such a pruning. For example, during the critical period observed here, does the VGLUT2+ afferents show a higher firing frequency that could account for no differences in the overall firing of 5-HT neurons? Are these two mechanisms working in a synergistic manner through which process of pruning is more directed to synapses that receive only VGLUT 1 as opposed to both VGLUT1 and VGLUT2? Some of these questions can really shed a positive light on the development of the Raphe-PFC pathway and how it is altered in disease. Thus, an experimental paradigm that would perform the same electrophysiological measurements in both these subpopulations coupled with a loss of function manipulation during animal development would bring the outcome of the research to a more conclusive story. Another area of questioning that can be resolved by such experiments is whether there is an intrinsic need for a much more pronounced glutamatergic input to 5-HT neurons from sub-cortical areas during post natal life than from cortical areas, and whether this synergistic approach to synaptic pruning is necessary to achieve the excitatory/inhibitory synaptic drives upon DRN neurons and how alteration of this pruning mechanism is involved in maladaptive mechanisms increasing the risk of developing mental disorders later in life.

We thank the reviewer for their comments. As shown before, the new electrophysiological data in the manuscript support the selective refinement of VGLUT1+ inputs on 5-HT neurons. We agree with the reviewer that studying functional consequences of the refinement upon the firing of glutamatergic afferent neurons to DRN 5-HT neurons, with or without loss of function manipulations, or to explore whether pruning alterations could be involved in maladaptive mechanisms increasing the risk of developing mental disorders, would be all very interesting and exciting topics to investigate. However, all these experiments will require very different electrophysiological setups and conditions, and we believe that they are beyond the scope of the present study. We now added in the discussion that: …future experiments involving a selective manipulation of this circuit, for instance by using optogenetics, will give us further mechanistic insight into the synaptic changes in this circuit during the refinement period….

Second decision letter

MS ID#: DEVELOP/2022/201121

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AUTHORS: Carla V Arganaraz, Tamara S Adjimann, Paula P Perissinotti, and Mariano Soiza-Reilly
ARTICLE TYPE: Research Report

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks. The two referee reports on this version are appended below. As you can see both reviewers were quite favorable. (Please note you have a typo on line 36 of the abstract (believe "shaping" should be "shape.") Thank you for submitting your paper to Development.

Reviewer 1

Advance summary and potential significance to field

This manuscript has been revised in a very satisfied way, excellent work.

Comments for the author

I have no additional comments or suggestions. It is acceptable for publication in Development.

Reviewer 2

Advance summary and potential significance to field

The manuscript has significantly gained after revision. In my opinion, all points raised by this revieser are sufficiently answered and the manuscript can be accepted in its present form.

Comments for the author

I have no additional comments