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Transneuronal Dpr12/DIP-δ facilitate compartmentalized dopaminergic innervation of Drosophila mushroom body axons

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

1st Editorial Decision 14th Jul 2020

Dear Oren,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the analysis interesting and support publication here. Many of the raised concerns can be addressed with a better explanation of how the experiments were done including driver lines used, timelines etc. Regarding point #1 raised by referee #3 - I think this is a good point. Do you have a good way to address this? I am happy to discuss the raised points further and maybe it would be most helpful to do so via phone or video. Let me know what works for you.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website:
https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

I thank you for the opportunity to consider your work for publication. Looking forward to discussing the revisions further with you

with best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please make sure you upload a letter of response to the referees' comments together with the revised manuscript.

Please also check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
http://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)

- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
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The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 12th Oct 2020.

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Referee #1:

How pre-and postsynaptic neurons within neural circuits organize themselves into defined zones during development continues to be poorly understood. During the past years, a new class of interacting immunoglobulin superfamily members, Dprs and DIPs, have come into focus as a diverse class of cell surface molecules mediating synaptic wiring specificity in Drosophila. However, despite intriguing combinatorial expression patterns, the evidence for a role in the formation of specific connections in the CNS had remained sparse, considering that the loss of these molecules has been described to cause considerable cell death prior to any synapse formation. This study of Bornstein and colleagues in the Oren Schuldiner lab focuses on the role of Dpr12 and DIP-delta during mushroom body circuit development. Their findings beautifully reveal that Dpr12 and DIP-delta are required for the zonal organization of mushroom body innervating neurons during pupal development. Dpr12 is expressed by intrinsic Kenyon cell (KC) gamma neurons and DIP-delta by a class of dopaminergic neurons (PAM DANs), innervating zones gamma 4 and 5. Extensive genetic data reveal that DIP-delta and Dpr12 mediate the interaction between specific PAMs and gamma KC neurons. In their absence, gamma KC neurons fail to extend to the distal tips and thus correct zones. The genetic approaches use state-of-the-art CRISPR/Cas9 and dual labeling approaches, recently developed by the laboratory. Phenotypes are clear and highly penetrant. The idea that PAM neurons could provide a template is intriguing, as one would not expect this normally from dopaminergic neurons. The study is thorough, very clearly written and all data support the conclusions. The manuscript also contains an engaging discussion, critically pinpointing key open questions about how DIPs and Dpr exert their function. I have very few suggestions for this convincing study, which I believe will be of wide interest to the circuit assembly community.

Specific comments:

1. The authors propose that DIP-delta is required and sufficient for Dpr12 localization. However, I am not sure whether the data make it possible to untangle a localization function from the role in controlling extensions into the gamma zones. A role in localization may need to tested in neurons whose connectivity is not changed.
2. Since in other parts of the CNS, cell loss and death is a concern, it would strengthen the study by providing direct evidence that this does not apply in their case.
3. A minor point is that genotypes should be consistently presented in italics.

Referee #2:

The work by Bornstein et al. entitled "Transneuronal Dpr12/DIP-δ interactions facilitate compartmentalized dopaminergic innervation of Drosophila mushroom body axons" describes a newly identified set of molecules that regulate the rewiring of the Drosophila mushroom body during metamorphosis.
Although it is known that the mushroom body is completely reconstructed during development (from the larval to the adult stage) the genetic basis is largely unknown. The work by Borstein and colleagues is therefore more than welcome and important. Particularly in light of the fact that the morphology and function of the adult mushroom body is at the centre of the latest scientific approaches focusing on brain functions in insect neurobiology. Likewise, the studies on insect mushroom bodies that identify this brain region as the insect learning and memory brain centre have a tradition going back decades. Therefore, the results of Bornstein and colleagues will very likely radiate beyond the Drosophila community.

In the following a list of concerns is given that I noticed while reading:

1) Summary
"The mechanisms controlling wiring of neuronal networks are largely unknown" I think these kinds of sentences are too strong and also have little content, since they are so unspecific that one can also delete them. Similarly - having also worked in this field for years - I also think that the content is not correct and ignores the work of many colleagues.

2) Introduction
"Despite its fundamental nature, the molecular and cellular mechanisms underlying development of neural circuits remain mostly poorly understood." Again, see comment 1). I think this statement is too strong.

3) Line 48:
"Given its unique development, connectivity and function, the Drosophila mushroom body (MB), ..." I don't think that unique is the right word. The logic of the argument is not clear to me either. If the MB was really unique, then it would not be desirable to analyze it, since no general developmental or functional principles could be derived. It is exactly the opposite, the MB is already very well analysed and offers insights that can be transferred to other brain regions in other organisms. The dopaminergic teaching neurons are a good example.

4) Line 65:
Would it be possible to provide a detailed description of the cellular configuration of the gamma 1-5 zones? How many input neuron, how many output neurons? In addition, not all terms are clearly

introduced. Input neuron, DANs, PAM, MBON. How many are there? Do the terms mean the same (DANs, PAM)? A figure might help. In addition the detailed anatomical EM description of gamma 5 was just published (Otto et al. Current Biology).

5) Line 79:
"These are innervated by MBONs and DANs in distinct zones that are different from the adult pattern of zonation". I'm not sure how different these zones are. There are 11 larval MB zones that all receive MBIN and MBON innervations. In the adult MB there are 16 zones in total also receiving MBIN and MBON innervations. For me that suggest that the basic organisation principle is the same. This can even be seen on the synaptic level using EM reconstructions. e.g. MBIN connect to KCs and also MBONs.

6) Figure 1 and Figure 2:
In both figures individual KCs are indicated but the calyx is missing. It is rather confusing to see these neurons without any input region.

7) Line 120:
"At this time we cannot conclude whether these Dprs are indeed not required for γ-KCs development or, alternatively, that the lack of phenotype is due to inherent redundancies of Dpr-DIP interactions" What about a limited efficiency of these RNAi lines. Did you check if the RNAi lines are really working?

8) Line 121 and 122.
Please delete the words "dramatic". This is not an objective scientific term. There is a clear structural change. I agree. But of course it is not dramatic.

9) Line 127
Do you see also other changes? For some dpr-RNAi combinations it seems that there is also an invervation of the alpha lobe (or even a faint alpha' lobe innervation). Is this a technical problem of increased background staining, or is this real due to effects on pruning of larval-born gamma KCs, so that they still have two axonal projections into the alpha and gamma lobe?

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Is this really an acute function? If I understand your experiments correctly, then all your genetic interference experiments cover the entire development and are not limited to the metamorphosis. The TARGET system, however, allows to clearly address the acute function of dpr12? This is nowadays standard in Drosophila studies.

11) Figure 1P
This is too small and hard to read.

12) Figure 2N
I see that the focus is on the gamma lobe - but again in yellow and green no real gamma KCs are shown. See also comment 6). So either show a correct KC scheme including the calyx, or focus only on the gamma lobes. In general I think that the important evaluation in N and I' and L' has to be increased. Especially given the huge size of the pictures from A-L. Also - why are there no "escapees" shown? It is a bit disturbing that always only the "STOP" at gamma 3-4 is shown, but in N and in the text also escapees are mentioned. Please also show the confocal data for them. Are these escapees are also seen when other KCs stop at gamma 3-4 or is this a brain specific effect. Or in other words, do you get exclusively "STOP" or escapees?

13) Line 167
Is it really the induction of growth? Or is it also possible that there are inhibitory signals that limit growth and usually gamma KCs can overcome this inhibition. However, in your mutant lines they loose this ability.

14) Line 206
Does the rescue also trigger misformation of gamma KCs? In both cases D and H I see strong GFP expression in the alpha lobes. Why is this the case? What does it mean?

15) Line 217
Maybe it makes sense to move the data described in that paragraph to the main body of the text. I think it is important. It would help to understand the next experiments.

16) Line 221
I think it would help to add one or two sentences about the cellular organisation of gamma4 and 5 here. See also comment 4) How many DANs innervate the zones? How many MBONs? Why do you ablate one gamma 4 MBON and PAM-DANs? What are PAM-DANs? How many? Does R58E02 include all input neurons for gamma4 and 5? I guess only some experts now these details, but most of the readers do not.

17) Figure 6
I don't understand the Fas2 staining in figure D. The staining should be comparable to figure 4G - however in this case the beta lobe is clearly smaller and altered. Why is that so - do you see that for other samples? What are the effects on other KC compartments?

18) Line 258
"dramatically" - see above 8). Please delete the word.

19) Line 266
Which neurons do you manipulate? Are these also PAMs? Again without a clear description of the DAN,PAM,MBON organisation of the gamma 3 lobe - these results are really hard to understand for non-experts. Also switching between DANs and PAM in the text is really hard to follow.

20) Line 281
What about the DANs that innervate gamma3? What happens if you repeat the experiments with MB441B-Gal4? I guess there is no change, or?

21) Line 292 and 295
Again the first sentence and the word "awesome" are a bit too strong or unscientific. Please change both. Awesome can be changed into comprehensive. I guess you rather refer to the nearly omnipotent power of the genetic tools available for Drosophila, or?

Referee #3:

This manuscript describes a series of genetic experiments investigating the role of Dpr12 and DIPdelta during neural circuit formation (including developmental rewiring). The experiments provide convincing data showing that both receptors are required for a specific targeting step where

regrowing gamma-neurons interact with a subset of dopaminergic neurons. The authors propose that direct receptor interactions mediate compartment specific contact in the distal subdomain of the gamma-neuron mushroom body lobe. This is a reasonable scenario but not well addressed experimentally.

Overall this an interesting analysis and technically well executed. Particularly the identification of the receptor pair and clear phenotypes (Fig1-4) is strong. More problematic is the second half of the paper, with substantial problems problems in the experimental description and figure documentation.

Critique points:

1) For me it remains unclear how Dpr12 and DIPdelta interactions specify the respective neurite interactions. There are only vague suggestions in the discussion. For me this is the main missing point and needs to be addressed directly. Either with better temporal resolution (i.e. how the phenotypes arise), ideally time lapse, or a simpler heterologous system where Dpr12 and DIPdelta interactions can be monitored directly.
2) For figures 5-7 many aspects of the analysis and experimental details are not sufficiently explained or missing. Many of the readers will not be experts of complex mushroom body development and the authors should therefore make an effort to explain important details to a broader audience. This relates to drivers, neuronal subtypes, timing etc. (see 3), but also conceptual issues (see 4).
3) Presumably, contact sites of dopaminergic neurites in zone 4 of the gamma lobe constitute synaptic contacts? What is pre- what is post-synaptic, or are these axo-axonic contacts. The authors imply a compartmentalized localization of DIPdelta, but given the complex 3-D structure of the mushroom body circuit it is not clear how this compartmentalization looks like. A full rendering of the respective dopaminergic projection and indication where DIPdelta is localized and where not would be very helpful.
4) Details of the dopaminergic or MBON neurons drivers are not described. What is MBON-g4>g1g2 (referred to in text (line 226/7) but not figure, - or differently in figure? What do profiling results referenced for by Coset et al. 2018 tell about DIPdelta expression?
5) Fig. 6 magenta and green channels should be shown separately.
6) What does CRE stand for in figure 7?
7) There should be a table giving the genotypes of all figure panels.

Smaller issues relate mainly to the text. In particular the discussion is hard to understand. For example (line 320: "... in dpr12 mutant animals PAM-DANs arrive to the right place, linger there for a while (~48h APF) and then eliminate their g4/5 innervations...". What do the authors ty to say here. When do the axons arrive, how long do they pause? And what does eliminate their innervation mean?

General response and major revisions:

We would like to thank the three reviewers for spending the time and effort for critical reading and constructive suggestions. We believe that thanks to their suggestions the revised manuscript is significantly improved. While we address the specific concerns of the reviewers in the point-by-point section below, we would first like to highlight the major changes in this revision:

1. As the reviewers highlighted, our depiction of the MB circuit anatomy was far from ideal. We have now used EM-derived skeletons (Scheffer et al., 2020) to generate new models of the MB circuitry as depicted in Figure 1P and Supplementary Movie 1.
2. To better characterize the γ-axon growth defect in dpr12 deficiencies, we analyzed *dpr12* RNAi-expressing single cell clones at 48hr APF compared to adult. We found that approximately 70% of single cell clones stop prematurely at both developmental stages, suggesting that Dpr12 deficient γ-axons never fully extend to the γ4/5 zones (rather than grow and retract).
3. We are excited to share a completely new experiment (Figures 8 and S8) that provides mechanistic insights onto Dpr-DIP interactions. We set out to investigate whether the *specific* interaction between Dpr12 and DIP-δ was required for γ4/5 zone formation, or, alternatively, whether other Dpr-DIP pairs could mediate circuit formation. To this end, we replaced DIP-δ with DIP-α in DIP-δ-expressing cells. We chose DIP-α because of its known interactions with Dpr6/Dpr10, which are both endogenously expressed in γ-KCs during metamorphosis. Remarkably, expressing DIP-α in DIP-δ-expressing PAM-DANs, in a *DIP-δ* homozygous mutant animal, completely suppressed the *DIP*-δ mutant phenotype and axons now extended to the end of the lobe (Figure 8A-B), indicating that DIP-α can fully compensate for the loss of DIP-δ. Furthermore, we showed that in this abnormal setup, in which DIP-δ is missing but γ-lobe morphology is seemingly normal, Dpr12 is no longer localized to the γ4/5 compartments as in the WT, but is instead diffused along the γ-lobe (Figure 8C-D). Additionally, overexpressing DIP-α in PAM-DANs, this time in a *dpr12* mutant animal in which DIP-δ is WT, also suppressed the *dpr12* mutant phenotype and facilitated full axon growth (Figure 8E-F). Together, these findings suggest that DIP-α does not function via Dpr12, but, most likely, via Dpr6/10, indicating that formation of the γ4/5 zones can be driven by matching Dpr-DIPs in γ-KCs and PAM-DANs, regardless of their specific identity. Finally, since Dpr/DIPs are IgSF proteins, expected to play, at least in principle, adhesive roles, we examined whether another adhesive interaction between γ-KCs and PAM-DANs could replace the Dpr12-DIP-δ interaction. However, we found that overexpressing FasII (a homophillic IgSF adhesion molecule that is endogenously expressed in γ-KCs) within DIP-δ-expressing cells was not sufficient to compensate for the loss of DIP-δ, and the γ4/5 zones failed to form (Figure S8C-D). Taken together, our findings suggest that a matching Dpr-DIP-mediated interaction between γ-KCs and PAM-DANs is required for the formation of the MB γ4/5 zones, via a

> mechanism that is not solely based on adhesion. This finding should facilitate the identification of the signaling mechanisms associated with Dpr-DIP interactions, not only during MB circuit formation but also in other neuronal systems.

Point-by-point response (in blue)**:**

Referee #1:

How pre-and postsynaptic neurons within neural circuits organize themselves into defined zones during development continues to be poorly understood. During the past years, a new class of interacting immunoglobulin superfamily members, Dprs and DIPs, have come into focus as a diverse class of cell surface molecules mediating synaptic wiring specificity in Drosophila. However, despite intriguing combinatorial expression patterns, the evidence for a role in the formation of specific connections in the CNS had remained sparse, considering that the loss of these molecules has been described to cause considerable cell death prior to any synapse formation. This study of Bornstein and colleagues in the Oren Schuldiner lab focuses on the role of Dpr12 and DIP-delta during mushroom body circuit development. Their findings beautifully reveal that Dpr12 and DIP-delta are required for the zonal organization of mushroom body innervating neurons during pupal development. Dpr12 is expressed by intrinsic Kenyon cell (KC) gamma neurons and DIP-delta by a class of dopaminergic neurons (PAM DANs), innervating zones gamma 4 and 5. Extensive genetic data reveal that DIP-delta and Dpr12 mediate the interaction between specific PAMs and gamma KC neurons. In their absence, gamma KC neurons fail to extend to the distal tips and thus correct zones. The genetic approaches use state-of-the-art CRISPR/Cas9 and dual labeling approaches, recently developed by the laboratory. Phenotypes are clear and highly penetrant. The idea that PAM neurons could provide a template is intriguing, as one would not expect this normally from dopaminergic neurons. The study is thorough, very clearly written and all data support the conclusions. The manuscript also contains an engaging discussion, critically pinpointing key open questions about how DIPs and Dpr exert their function. I have very few suggestions for this convincing study, which I believe will be of wide interest to the circuit assembly community.

We thank the reviewer for his/her kind words and support.

Specific comments:

1. The authors propose that DIP-delta is required and sufficient for Dpr12 localization. However, I am not sure whether the data make it possible to untangle a localization function from the role in controlling extensions into the gamma zones. A role in localization may need to tested in neurons whose connectivity is not changed.

We thank the reviewer for this important insight. In principle, the data from larvae (Figure 6A) already suggests that Dpr12 localization depends on DIP-δ regardless of axon

growth. That said, the reviewer's comment motivated us to examine this further. In a completely new experiment (detailed above in "major changes"), we performed a replacement experiment in which we expressed, in PAM-DANs, DIP-α instead of DIP-δ. Remarkably, this completely suppressed the growth defect of *DIP-δ* mutants (Figure 8). In this genetic background, where DIP-δ is missing but axon growth is seemingly normal, Dpr12-GFP is diffusely localized along the γ-lobe. Thus, our results suggest that the localization of Dpr12 depends on its interaction with DIP-δ, independently of axon growth.

2. Since in other parts of the CNS, cell loss and death is a concern, it would strengthen the study by providing direct evidence that this does not apply in their case.

The reviewer is absolutely right. The data presented in Figure S6C,F, arguing against the involvement of cell death-related mechanism, is now better referenced within the text.

3. A minor point is that genotypes should be consistently presented in italics.

We apologize for this inadvertence and have amended the manuscript according to the reviewer's comment.

Referee #2:

The work by Bornstein et al. entitled "Transneuronal Dpr12/DIP-δ interactions facilitate compartmentalized dopaminergic innervation of Drosophila mushroom body axons" describes a newly identified set of molecules that regulate the rewiring of the Drosophila mushroom body during metamorphosis.
Although it is known that the mushroom body is completely reconstructed during development (from the larval to the adult stage) the genetic basis is largely unknown. The work by Borstein and colleagues is therefore more than welcome and important. Particularly in light of the fact that the morphology and function of the adult mushroom body is at the centre of the latest scientific approaches focusing on brain functions in insect neurobiology. Likewise, the studies on insect mushroom bodies that identify this brain region as the insect learning and memory brain centre have a tradition going back decades. Therefore, the results of Bornstein and colleagues will very likely radiate beyond the Drosophila community.

We thank the reviewer for highlighting the importance of our study.

In the following a list of concerns is given that I noticed while reading:

1) Summary
"The mechanisms controlling wiring of neuronal networks are largely unknown" I think

these kinds of sentences are too strong and also have little content, since they are so unspecific that one can also delete them. Similarly - having also worked in this field for years - I also think that the content is not correct and ignores the work of many colleagues.

To more accurately reflect the knowledge in the field, we now modified this sentence to 'The mechanisms controlling wiring of neuronal networks are not completely understood'.

2) Introduction
"Despite its fundamental nature, the molecular and cellular mechanisms underlying development of neural circuits remain mostly poorly understood." Again, see comment 1). I think this statement is too strong.

We changed to 'our knowledge remains incomplete'.

3) Line 48:
"Given its unique development, connectivity and function, the Drosophila mushroom body (MB), ..." I don't think that unique is the right word. The logic of the argument is not clear to me either. If the MB was really unique, then it would not be desirable to analyze it, since no general developmental or functional principles could be derived. It is exactly the opposite, the MB is already very well analysed and offers insights that can be transferred to other brain regions in other organisms. The dopaminergic teaching neurons are a good example.

We thank the reviewer for this comment, and now changed 'unique' to 'well-studied'.

4) Line 65:
Would it be possible to provide a detailed description of the cellular configuration of the gamma 1-5 zones? How many input neuron, how many output neurons? In addition, not all terms are clearly introduced. Input neuron, DANs, PAM, MBON. How many are there? Do the terms mean the same (DANs, PAM)? A figure might help. In addition the detailed anatomical EM description of gamma 5 was just published (Otto et al. Current Biology).

We thank the reviewer for highlighting the importance of detailed and accurate schematics. His/her comments motivated us to explore the newly available hemibrain EM data generated by Janelia Research Campus (Scheffer et al., 2020). We have made new models based on these EM reconstructions (Figure 1P and supplementary Movie 1), and updated our schematics to reflect the fact that the cell body of MBON-γ4>γ1γ2 is situated on the contralateral hemisphere (Figure 1Q).
Additionally, on the basis of detailed characterization of the MB circuit by Li and colleagues (2020), we have now added more information to better introduce in the text

the anatomy of the MB circuit (including precise numbers). We also now relate to the further subdivision of γ5 DANs subtypes as discovered by Otto et al.
Finally, to avoid confusion, we now consistently write 'PAM-DANs' throughout the manuscript.

5) Line 79:
"These are innervated by MBONs and DANs in distinct zones that are different from the adult pattern of zonation". I'm not sure how different these zones are. There are 11 larval MB zones that all receive MBIN and MBON innervations. In the adult MB there are 16 zones in total also receiving MBIN and MBON innervations. For me that suggest that the basic organisation principle is the same. This can even be seen on the synaptic level using EM reconstructions. e.g. MBIN connect to KCs and also MBONs.

We now reworded this sentence, to better reflect that the organizational principles are maintained but the zonation itself is remodeled.

6) Figure 1 and Figure 2:
In both figures individual KCs are indicated but the calyx is missing. It is rather confusing to see these neurons without any input region.

We thank the reviewer for this clarification. We have now added the calyx to our schematics. However, we still left out the calyces from the Z-projections, to allow the reader to focus on the axonal defects. Of note, slight differences in brain orientation can result in large presumed differences of calycal localization, and in most cases the calyx appears behind the axons, precluding their clear visualization.

7) Line 120:
"At this time we cannot conclude whether these Dprs are indeed not required for γ-KCs development or, alternatively, that the lack of phenotype is due to inherent redundancies of Dpr-DIP interactions" What about a limited efficiency of these RNAi lines. Did you check if the RNAi lines are really working?

We thanks the reviewer for this comment, indeed limited RNAi efficiency is an additional explanation, as was now added to the text.

8) Line 121 and 122.
Please delete the words "dramatic". This is not an objective scientific term. There is a clear structural change. I agree. But of course it is not dramatic.

We now replaced the word 'dramatic' with more subtle and objective terms, such as 'pronounced defect' and 'markedly reduced'.

9) Line 127
Do you see also other changes? For some dpr-RNAi combinations it seems that there is

also an invervation of the alpha lobe (or even a faint alpha' lobe innervation). Is this a technical problem of increased background staining, or is this real due to effects on pruning of larval-born gamma KCs, so that they still have two axonal projections into the alpha and gamma lobe?

Indeed it seems that in Dpr10 RNAi there is a very mild defect in pruning of the γ axons, resulting in vertically projecting GFP axons outside the fasciculated α lobe. While this may be interesting by itself (and now annotated in the legend of Supplementary Figure 1), it is outside the scope of this paper and may distract the reader.
Related to this comment: while the R71G10 driver is consistently expressed in γ–KCs, it is also expressed in α/β-KCs in a stochastic manner, for reasons that remain unknown. This accounts for the GFP expression in the α/β lobes, as we now clarified in the main text and in the figure legend. In addition to providing new EM-based schematics, we now also outlined the α/β and γ lobes in Figures 1 (R71G10-Gal4) and 4 (R71G10-QF2). We also added a new table (Supplementary Table 3) that describes all the drivers used in the current study.

10) Line 143
Is this really an acute function? If I understand your experiments correctly, then all your genetic interference experiments cover the entire development and are not limited to the metamorphosis. The TARGET system, however, allows to clearly address the acute function of dpr12? This is nowadays standard in Drosophila studies.

We agree with the reservation of this reviewer about the acute function of Dpr12. We have now added a sentence to better reflect this limitation ('Importantly, its requirement during metamorphosis does not rule out additional roles of Dpr12 in other developmental stages').

11) Figure 1P
This is too small and hard to read.

We now enlarged the font (now Figure 1O).

12) Figure 2N
I see that the focus is on the gamma lobe - but again in yellow and green no real gamma KCs are shown. See also comment 6). So either show a correct KC scheme including the calyx, or focus only on the gamma lobes. In general I think that the important evaluation in N and I' and L' has to be increased. Especially given the huge size of the pictures from A-L. Also - why are there no "escapees" shown? It is a bit disturbing that always only the "STOP" at gamma 3-4 is shown, but in N and in the text also escapees are mentioned. Please also show the confocal data for them. Are these escapees are also seen when

other KCs stop at gamma 3-4 or is this a brain specific effect. Or in other words, do you get exclusively "STOP" or escapees?

Based on these important comments, we performed the following changes:

1. We present and trace a brain containing several Dpr12 RNAi-expressing single-cell clones, in which one axon grew fully, while the others stopped prematurely, indicating that this is not a brain-specific effect, as is now explained in the text.
2. We modified the graph (now in panel M) to clearly focus on the γ lobe, and to enable better representation (larger fonts).

13) Line 167
Is it really the induction of growth? Or is it also possible that there are inhibitory signals that limit growth and usually gamma KCs can overcome this inhibition. However, in your mutant lines they loose this ability.

We address these important points in the discussion section, which we now refer the reader to.

14) Line 206
Does the rescue also trigger misformation of gamma KCs? In both cases D and H I see strong GFP expression in the alpha lobes. Why is this the case? What does it mean?
As explained in comment 9, while the R71G10-QF2 driver is consistently expressed in γ – neurons, it is also expressed in α/β neurons in a stochastic manner, for reasons that remain unknown, and may be related to transgenic load. This was now properly explained in the text and in the relevant figure legends.

15) Line 217
Maybe it makes sense to move the data described in that paragraph to the main body of the text. I think it is important. It would help to understand the next experiments.

Thanks for the suggestion - we now moved the RNAi experiments to the main Figure (now in Figure 4).

16) Line 221
I think it would help to add one or two sentences about the cellular organisation of gamma4 and 5 here. See also comment 4) How many DANs innervate the zones? How many MBONs? Why do you ablate one gamma 4 MBON and PAM-DANs? What are PAM-DANs? How many? Does R58E02 include all input neurons for gamma4 and 5? I guess only some experts now these details, but most of the readers do not.

In response to comment 4, we have now generated more detailed schematics of MB connectivity focusing on the γ4/5 zones, based on the hemibrain EM reconstructions (Scheffer et al., 2020). We think this is an important addition to explain the cellular organization that is relevant to this study and thank the reviewer for this.

The precise numbers and cellular architecture of the adult MB are currently being explored by several groups (e.g., Aso, Rubin, Waddell). Therefore, we feel that imposing more quantitative information on the reader in the context of this study is unnecessary. Finally, it is currently unknown whether R58E02-Gal4 labels all PAM-DANs, and given its somewhat variable expression between animals, we think this might be difficult to conclusively determine, and regardless is beyond the scope of this study.

17) Figure 6
I don't understand the Fas2 staining in figure D. The staining should be comparable to figure 4G - however in this case the beta lobe is clearly smaller and altered. Why is that so - do you see that for other samples? What are the effects on other KC compartments?

We thank the reviewer for his/her sharp perception – indeed, in approximately 40% of the *dpr12* homozygous mutant brain we observe defects in the full extension of the β lobe. This was observed only in *dpr12* homozygous mutants and not in the *Dpr12* RNAi. Because the morphology of the β lobe is rescued upon overexpression of Dpr12 within the γ neurons (data not shown), we conclude that this is most likely a non cell-autonomous defect. While this is interesting on its own, it is beyond the scope of the current paper. The β lobe morphological defect was originally mentioned in the legend of Supplementary Figure 1D but is now more fully explained, and also referred to in the legend of Figure 6.

18) Line 258
"dramatically" - see above 8). Please delete the word.

Done.

19) Line 266
Which neurons do you manipulate? Are these also PAMs? Again without a clear description of the DAN,PAM,MBON organisation of the gamma 3 lobe - these results are really hard to understand for non-experts. Also switching between DANs and PAM in the text is really hard to follow.

We agree with the reviewer that this was confusing, we now changed the name to 'PAM-DANs' throughout the text and figures.

20) Line 281
What about the DANs that innervate gamma3? What happens if you repeat the experiments with MB441B-Gal4? I guess there is no change, or?

The suggested experiment is unfortunately genetically challenging (since MB441B is a split Gal4 on both the 2nd and 3rd chromosomes, and the 2nd chromosome must be

homozygous mutant for dpr12). Since we could not find another, more genetically suitable driver for PAM-DANs targeting γ3, we tested the expression of MB083C, expressed in the MBON-γ3β'1 (which innervates both the γ3-lobe and the β'1-lobe). As we expected, when we focus on the γ-lobe, we see no detectible change in the extent or pattern of the MBON innervation of γ3 between dpr12 heterozygote brains (with WT-like morphology) and homozygous mutant brains (which lack the γ4-5 zones). This data is now included in Supplementary Figure 7.

21) Line 292 and 295
Again the first sentence and the word "awesome" are a bit too strong or unscientific. Please change both. Awesome can be changed into comprehensive. I guess you rather refer to the nearly omnipotent power of the genetic tools available for Drosophila, or?

We now changed 'awesome' to 'comprehensive'.

Referee #3:

This manuscript describes a series of genetic experiments investigating the role of Dpr12 and DIPdelta during neural circuit formation (including developmental rewiring). The experiments provide convincing data showing that both receptors are required for a specific targeting step where regrowing gamma-neurons interact with a subset of dopaminergic neurons. The authors propose that direct receptor interactions mediate compartment specific contact in the distal subdomain of the gamma-neuron mushroom body lobe. This is a reasonable scenario but not well addressed experimentally.

Overall this an interesting analysis and technically well executed. Particularly the identification of the receptor pair and clear phenotypes (Fig1-4) is strong. More problematic is the second half of the paper, with substantial problems problems in the experimental description and figure documentation.

We thank the reviewer for his/her kind words and support.

Critique points:

1) For me it remains unclear how Dpr12 and DIPdelta interactions specify the respective neurite interactions. There are only vague suggestions in the discussion. For me this is the main missing point and needs to be addressed directly. Either with better temporal resolution (i.e. how the phenotypes arise), ideally time lapse, or a simpler heterologous system where Dpr12 and DIPdelta interactions can be monitored directly.

The reviewer seems to raise a key issue that relates to our incomplete mechanistic understanding of the Dpr-DIP interactions leading to the circuit formation within γ4/5 zones. He/she is absolutely correct, but it is important to mention that the many high-

impact papers published on Dpr-DIP interactions have also failed in identifying the precise mechanisms by which they function. Thus, depicting the precise mechanistic interactions between these molecules will likely be the topic of many future studies. That said, we have tried to explore, or have considered several directions in this regard:

1. The biochemical binding interaction between Dpr12 and DIP-δ has been characterized in detail by several groups (Ozkan et al., 2013; Carillo et al., 2015; Cosmanesco et al., 2018). We therefore do not understand the potential contribution of a heterologous system.
2. While we have previously established an ex-vivo brain culture system (Rabinovich et al. 2015), performing time-lapse imaging of WT and mutant γ-axons regrowing into the adult lobe is extremely challenging. That said, we did aim to achieve better temporal resolution in many of our key experiments, as follows:
    a. For Dpr12-GFP and DIP-δ-GFP localization experiments, we added an additional time point (72hr APF, presented in Figure S3).
    b. To understand how the wiring defects presented in Figure 7 arise, we attempted to explore additional time points throughout metamorphosis. Unfortunately, our experiments revealed that the specific PAM-DAN-Gal4s are not expressed in early pupal stages.
    c. In contrast to the specific PAM-DAN-Gal4s mentioned in point b, the DIP-δ-Gal4 that we have generated robustly labels PAM-DANs that target the γ4/5 zones throughout development. Alas, it also labels many other cells (as shown is Figure S5). We therefore attempted to expand our mosaic analyses of DIP-δ-Gal4 in WT (as shown in Figure 5) and *DIP-δ* homozygous mutants throughout key time points during development. The broad expression of DIP-δ-Gal4 unfortunately precluded us from interpreting the data we obtained. Our means of identifying the γ4/5 PAM-DANs within DIP-δ-Gal4 MARCM clones is by the virtue of their typical innervation. In *DIP-δ* mutants, this innervation is disrupted and thus we cannot conclusively identify these neurons.
    Given points b and c, in order to study the development of the PAM-DANs, there is an urgent need to develop new tools to label specific neurons during development (not only PAM-DANs, e.g., see our APL study, Mayseless et al. 2018). We are in the process of exploiting the DIP-δ-Gal4 and intersectional Gal4 genetics to achieve this in the future.
    d. Finally, but perhaps most importantly, in order to discriminate between failure of γ-axons to extend into the γ4/5 zones vs. extension followed by retraction, we quantified Dpr12 RNAi-expressing single-cell clones at 48hr APF (Figure 2N). Remarkably, the percentage of axons prematurely stopping at 48hr APF was comparable to that in the adult. These data suggest that γ-axons lacking Dpr12 are unable to elaborate into the γ4/5 zones.
3. Like the reviewer, we were also interested in gaining a mechanistic understanding of the Dpr12-DIP-δ interaction in mediating γ4/5 zone formation.

We therefore performed the replacement experiments that are described, in detail, in the above 'major revisions' section, and presented in the completely new Figures 8 and S8. Taken together, the conclusion of these experiments suggests that the identity of the Dpr-DIP molecules is not important, as long as a matching pair is expressed in the relevant neurons (in the case of γ4/5 zone formation – γ-KCs and PAM-DANs). This finding should facilitate the identification of the signaling mechanisms associated with Dpr-DIP interactions, not only during MB circuit formation but also in the visual system and motoneurons.

2) For figures 5-7 many aspects of the analysis and experimental details are not sufficiently explained or missing. Many of the readers will not be experts of complex mushroom body development and the authors should therefore make an effort to explain important details to a broader audience. This relates to drivers, neuronal subtypes, timing etc. (see 3), but also conceptual issues (see 4).

We now added a table of all the drivers used in this study, describing the cells in which they are expressed in different developmental stages (Supplementary Table 3). In addition, we used the EM hemibrain data (Scheffer et al., 2020) in order to generate new models of the MB architecture, focusing on the γ4/5 zones (Figure 1P and Supplementary Movie 1). We believe that these models, plus more detailed anatomic analyses (in Figure 3), as well as improved figure annotations, should assist the reader in better understanding the phenotypic defects. See also response to comments 3 and 4.

3) Presumably, contact sites of dopaminergic neurites in zone 4 of the gamma lobe constitute synaptic contacts? What is pre- what is post-synaptic, or are these axo-axonic contacts. The authors imply a compartmentalized localization of DIPdelta, but given the complex 3-D structure of the mushroom body circuit it is not clear how this compartmentalization looks like. A full rendering of the respective dopaminergic projection and indication where DIPdelta is localized and where not would be very helpful.

To the best of our understanding, how, precisely, dopaminergic signaling modulates the synaptic transmission between KCs and MBONs is incompletely understood. Manuscripts from experts in the field (e.g., Rubin lab, Aso lab) refer to the dopaminergic arbors as 'processes' without committing to pre- or post-synapses. Recent EM data has discovered reciprocal synaptic structures between KCs and DANs. Therefore, defining either one as an axon or dendrite is not straightforward. Humbly, we decided to stick with the term 'processes' without going into more specific definitions.

Regarding the compartmentalized localization of DIP-δ in the γ4-5 zones: perhaps what the reviewer is referring to is our lack of subcellular localization resolution. This will require the generation of new genetic tools combined with super-resolution and therefore is beyond the scope of this study. However, to better demonstrate the

localization of the DIP-δ-GFP fusion protein, as shown in Figure 3, we now added transverse slices, which clearly demonstrate the colocalization of DIP-δ with the γ4 zone (as determined by the well-characterized MBONγ4>γ1γ2; Figure 3D,H).
In addition, we added new images and a movie (Figure 1P, Supplementary Movie 1), which are based on the publically available EM-traces (Scheffer et al., 2020), that display the 3D architecture of the γ4/5 MB circuitry in greater detail.

4) Details of the dopaminergic or MBON neurons drivers are not described. What is MBON-g4>g1g2 (referred to in text (line 226/7) but not figure, - or differently in figure? What do profiling results referenced for by Coset et al. 2018 tell about DIPdelta expression?

MBON-γ4>γ1γ2, also called MBON-05, is a specific type of MBON, one of two MBON types that innervate the γ4-zone (the other being MBON-γ4γ5 or MBON-21). We now changed the text and figure legends to consistently refer to this MBON by its full name (within the figures themselves we still often refer to it as MBON-γ4 for simplicity, but this is always clarified in the relevant legend). Furthermore, a list of neurons used to create the EM-based model in Figure 1P is available in Supplementary Table 4.
Croset et al. showed that DIP-δ is enriched in PAM-DANs, and in fact even suggested its use as a new marker for PAM-DANs. We thank the reviewer for highlighting this issue, and now modified the text to clarify this point.

5) Fig. 6 magenta and green channels should be shown separately.

We now added a separate display of the channels in Figure 6.

6) What does CRE stand for in figure 7?

Cre stands for Crepine, a neuropil that surrounds the medial MB lobes and functions (along with other adjacent neuropils) as a convergence zone for DAN dendrites and MBON axons (Aso et al. 2014a). This was now clarified in the text and the relevant figure legend.

7) There should be a table giving the genotypes of all figure panels.

A full list of the genotypes of all figure panels is given in the supplementary material.

8) Smaller issues relate mainly to the text. In particular the discussion is hard to understand. For example (line 320: "... in dpr12 mutant animals PAM-DANs arrive to the right place, linger there for a while (~48h APF) and then eliminate their g4/5 innervations...". What do the authors ty to say here. When do the axons arrive, how long do they pause? And what does eliminate their innervation mean?

Given our limited ability to label PAM-DANs during development (see point number 2c in comment 1 above), we cannot provide specific times. However, we agree with the reviewer that this sentence was confusing, and now reworded it as follows: ‘Interestingly, our DIP-δ localization experiments suggest that in dpr12 mutant animals, PAM-DANs arrive to the right place (the future γ4/5 zones) during larval development, maintain their processes at least until 48hr APF, but eventually (at a yet unknown time point) eliminate or remodel their γ4/5 innervations, while maintaining and even strengthening/broadening other connections in this vicinity’.

1st Revision - Editorial Decision 22nd Jan 2021

Dear Oren,

Thanks for submitting your revised manuscript. Your study has now been seen by the original referees. As you can see below they appreciate the introduced changes and support publication here. Referee #3 would like a more careful description and discussion about on the role of Dpr12/DIPdelta interaction. This can be done with text changes.

When you submit the revised manuscript will you also please take care of the following points:

- Please fix the number of keywords - you can only have 5

- Please add a Data availability section (at the moment part of the ack. section) => place it after the Materials and methods and before Acknowledgements and add the accession numbers for RNA-seq data to this section. Please also add this info to author checklist.

- COI should be a separate section as well (now part of the acknowledgements)

- We are missing an author contribution section

- Please double check the reference section some references have no issue or page numbers (ie Bilz et al)

- We don't allow data not shown (pgs 6,10,14) either include it or re-phrase

- The ORCID ID is missing for Meltzer

- The images in Fig 7A-D are reused in S6A, B, D, E - correct? If so please say this clearly in the figure legend of S6

- Is Fig 2O reused in 7E - please take a look?

- Can you make sure that the Fig S5D-F green channel in magnifications match the origin images?

- You can only have 5 EV figure figures - the other figures would have to be added to the appendix. The figure legends for the EV figures should be in the main text while the figure legends for the appendix figures in the appendix. For the appendix please make sure that it has a ToC and page numbered

- The EV figure, Appendix figures/tables and movie callouts need to be corrected. Please check author guidelines.

- Please add a proper M&M section to the main manuscript (now in the appendix).

- For the reagents table (now part of the appendix) please upload as a separate table and add a callout to this in M&M

- The genotypes listed for each figure in the appendix can you make sure that there is a call out to this in the text - maybe in the figure legends or the M&M

- Tables S1 + S2 should be named Dataset EV1 + EV2. Please add the legends as a separate tab

- The movie legends need to be zipped with the respective movies

- Summary should be corrected to Abstract and please make sure that the MS sections are in the right order.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

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- I have asked our publisher to do their pre-publication checks on the paper. They will send me the file within the next few days. Please wait to upload the revised version until you have received their comments.

When you resubmit your revised version please include a point-by-point response also to the editorial points

That should be all let me know if you have any further questions.

Congratulations on a nice study

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

Instructions for preparing your revised manuscript:

Please check that the title and abstract of the manuscript are brief, yet explicit, even to non-specialists.

When assembling figures, please refer to our figure preparation guideline in order to ensure proper formatting and readability in print as well as on screen:
https://bit.ly/EMBOPressFigurePreparationGuideline

IMPORTANT: When you send the revision we will require
- a point-by-point response to the referees' comments, with a detailed description of the changes made (as a word file).
- a word file of the manuscript text.
- individual production quality figure files (one file per figure)

- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

Please remember: Digital image enhancement is acceptable practice, as long as it accurately represents the original data and conforms to community standards. If a figure has been subjected to significant electronic manipulation, this must be noted in the figure legend or in the 'Materials and Methods' section. The editors reserve the right to request original versions of figures and the original images that were used to assemble the figure.

Further information is available in our Guide For Authors:
https://www.embopress.org/page/journal/14602075/authorguide

The revision must be submitted online within 90 days; please click on the link below to submit the revision online before 22nd Apr 2021.

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

This excellent study of Bornstein et al. provides novel insights into the role of Dpr and DIPs in zonal circuit assembly in the mushroom bodies of Drosophila. I have been very favorable of the earlier version of the study, and am even more supportive for this new strengthened version of the manuscript. The novel data provide convincing evidence that the localization of Dpr12 depends on the interaction with DIP-delta. All in all, the authors addressed all my concerns and I would like to strongly recommend this manuscript for publication.

A very minor point is the mentioning of « data not shown » e.g. line 151, which is no longer acceptable in publications, I believe.

Referee #2:

The manuscript has been completely revised and is much improved. I am satisfied with the responses to my comments and those of the other two reviewers and congratulate the authors on their excellent work.

Referee #3:

The authors revised the manuscript with text edits, helpful additional information, and new results. With the exception of a key point (see below) most critique points have been addressed and improved the manuscript.

However, the key concern and question how Dpr12 and DIPdelta interactions specify the respective neurite interactions in the mushroom body was not addressed. If I understand correctly, the authors suggest that this is difficult and currently not possible.
If that is the case than the manuscript text needs to be adapted and should explain precisely what the experiments addressed and what this reveals regarding Dpr12/DIPdelta functions or potential interactions.
To explain this point better I elaborate a bit more:
The authors present data on the expression of DIPdelta using a gene-trap line. For example data in figure 3 E-H show expression in zone gamma4, but not other gamma zones. Data from multiple figures/panels suggest that there is no expression of DIPdelta in zone gamma 5, where the Dpr12 expressing axons terminate (see figure 2M). Therefore, it is unclear why the authors state: "...We found that DIP-δ-GFP ... localized, similarly to Dpr12, to the γ4/5 zones." Stated as such it is incorrect or misleading. In fact the data make it difficult to explain the mutant phenotype based on the proposed direct Dpr12/DIPdelta interactions.
Why do Dpr12 expressing axons extend beyond the zone where DIPdelta is localized (i.e. zone 4)? This is not consistent with high affinity adhesive interactions (as suggested by several biochemical studies). The authors need to clarify this in the manuscript text and discuss it critically.
To me it remains entirely unclear what Dpr12/DIPdelta interactions specify in this experimental system. Related, looking at the summary (and drawing) in figure 7 G/H it seems clear that dpr12 mutants have a lot more defects than just the zone4/5 compartmentalization. This needs to be discussed.
It is unclear why the authors state: "...DIP-δ functions in a subset of dopaminergic neurons that wire with γ-neurons within the γ4/5 zone." What is the evidence that DIPdelta is expressed in zone 5? What does wire mean in this context? Synaptic contacts? Do the authors have evidence for this?
Further: "...We found that the immunoglobulin superfamily protein Dpr12 is cell-autonomously required in γ-neurons for their developmental regrowth into the distal γ4/5 zones, where both Dpr12 and its interacting protein, DIP-δ, are enriched...?
I agree that the data are clearly showing that Dpr12 is required in gamma-neurons. But it is quite confusing regarding the Dpr12-DIPdelta interactions. It is misleading to suggest that both proteins are "enriched" at the same location. I assume the authors want to propose that the projecting gamma-neurons have Dpr12 on their axons which can bind to DIPdelta (in trans, across cell membranes!), which is expressed/presented by target neurites of dopaminergic neurons.
Is Dpr12 really required for "developmental regrowth"? Or just for axon growth after regrowth was initiated?

Point-by-point reply to editorial requests and reviewers

Dear Karin,
Thanks for your email and positive feedback.
Please find our point-by-point reply to the editorial requests and reviewers' comments.

- Please fix the number of keywords - you can only have 5

Done, we now modified the keywords to include only 5.

- COI should be a separate section as well (now part of the acknowledgements)

The COI now appears as a separate section.

- We are missing an author contribution section

We now added an author contribution section.

- Please double check the reference section some references have no issue or page numbers (ie Bilz et al)

We now added all the missing details in the references.

- We don't allow data not shown (pgs 6,10,14) either include it or re-phrase

We now modified the text in all the relevant places.

- The ORCID ID is missing for Meltzer.

We now added the ORCID ID for Meltzer.

- The images in Fig 7A-D are reused in S6A, B, D, E - correct? If so please say this clearly in the figure legend of S6

We now clearly state in the figure legend of EV Figure 5 (formerly S6A) that this is detailed analyses of the confocal z-projections in Figure 7A-F.

- Is Fig 2O reused in 7E - please take a look?

Indeed, the same figure was accidentally used twice, we apologize for this confusion and thank you for noticing. The image in 2O was now replaced with a different image.

- Can you make sure that the Fig S5D-F green channel in magnifications match the origin images?

The magnified images are slightly different than the original ones since they only include a subset of the z-projection of the original image, which is restricted to the slices that contain the γ-lobe. This was now better annotated within the figure (‘sub-z-projection’) and also explained in the legend.

- You can only have 5 EV figure figures - the other figures would have to be added to the appendix. The figure legends for the EV figures should be in the main text while the figure legends for the appendix figures in the appendix. For the appendix please make sure that it has a ToC and page numbered.

As you kindly agreed, the manuscript now has 6 EV figures (legends within the main text), and in addition two appendix figures (figures+ legends within the appendix).

- The EV figure, Appendix figures/tables and movie callouts need to be corrected. Please check author guidelines.

All callouts were corrected throughout the manuscript.

- Please add a proper M&M section to the main manuscript (now in the appendix).

We now moved the materials and methods section to the main text.

- For the reagents table (now part of the appendix) please upload as a separate table and add a callout to this in M&M

We now separated it, named it Reagents Table, and added a callout in the beginning of the materials and methods section.

- The genotypes listed for each figure in the appendix can you make sure that there is a call out to this in the text - maybe in the figure legends or the M&M

We now added a callout to the list of genotypes (now within the appendix) in the materials and methods section, under ‘experimental model’.

- Tables S1 + S2 should be named Dataset EV1 + EV2. Please add the legends as a separate tab

We now renamed the tables and added the legends in separate excel sheets.

- The movie legends need to be zipped with the respective movies

Each movie was now zipped with its legends (as a word document).

- Summary should be corrected to Abstract and please make sure that the MS sections are in the right order.

We now corrected ‘summary’ to ‘abstract’ and changed the section order to comply with your guidelines.

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With best wishes

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Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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- individual production quality figure files (one file per figure)
- a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14602075/authorguide).
- Expanded View files (replacing Supplementary Information)
Please see out instructions to authors
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Perhaps we were misunderstood. We never claimed that the experiments were too difficult for us to do. Indeed, having done many of these experiments, it is now clear that many reagents (mostly Gal4 driver lines) are lacking. Specifically, while distinct Gal4 drivers exist to label adult neuronal populations, many are not specific or not expressed at all during development, thus precluding a meaningful interpretation.

To explain this point better I elaborate a bit more:
The authors present data on the expression of DIPdelta using a gene-trap line. For example data in figure 3 E-H show expression in zone gamma4, but not other gamma zones. Data from multiple figures/panels suggest that there is no expression of DIPdelta in zone gamma 5, where the Dpr12 expressing axons terminate (see figure 2M). Therefore, it is unclear why the authors state: "...We found that DIP-δ-GFP ... localized, similarly to Dpr12, to the γ4/5 zones." Stated as such it is incorrect or misleading. In fact the data make it difficult to explain the mutant phenotype based on the proposed direct Dpr12/DIPdelta interactions.
Why do Dpr12 expressing axons extend beyond the zone where DIPdelta is localized (i.e. zone 4)? This is not consistent with high affinity adhesive interactions (as suggested by several biochemical studies). The authors need to clarify this in the manuscript text and discuss it critically.

We apologize for the suboptimal figure representation that led the reviewer to misunderstand our data. While it is true that DIP-δ is much more strongly expressed in γ4 than in γ5, both the DIP-δ-GFP fusion protein, as well as the DIP-δ-Gal4 experiments, clearly demonstrate that it is also present in γ5. We have now added

insets in Figure 3 and quantifications in Appendix Figure 1 to demonstrate this point, and added explanatory text in the appropriate locations.

To me it remains entirely unclear what Dpr12/DIPdelta interactions specify in this experimental system. Related, looking at the summary (and drawing) in figure 7 G/H it seems clear that dpr12 mutants have a lot more defects than just the zone4/5 compartmentalization. This needs to be discussed.

A broad look at the DIP-δ-Gal4 expression (EV Figure 4D-F) indeed confirms the reviewer's notion that it is likely to be required in other processes in the CNS. While in this paper, we focus mostly on γ4-5 formation, we have also alluded to the potentially broader functions of this interaction in the sections describing the DIP-δ-Gal4 expression, as well as in our analysis of the broad structural changes in Dpr12 mutants – specifically regarding the changes in the Crepine neuropil (Figure 7G-F) – now more extensively discussed in the text.

It is unclear why the authors state: "...DIP-δ functions in a subset of dopaminergic neurons that wire with γ-neurons within the γ4/5 zone." What is the evidence that DIPdelta is expressed in zone 5? What does wire mean in this context? Synaptic contacts? Do the authors have evidence for this?

The experiments that led us to conclude that the major DIP-δ contribution within the γ lobe comes from its expression in PAM-DANs are described in Figure 5. See our comment above about DIP-δ expression within the γ5 zone. The precise nature of the wiring interactions between γ-KCs and DANs remains unclear, despite many genetic and EM studies, and thus is beyond the scope of this manuscript. Important to note that due to lack of direct evidence of synaptic connections, we refrained from using this term throughout the manuscript.

Further: "...We found that the immunoglobulin superfamily protein Dpr12 is cell-autonomously required in γ-neurons for their developmental regrowth into the distal γ4/5 zones, where both Dpr12 and its interacting protein, DIP-δ, are enriched...?
I agree that the data are clearly showing that Dpr12 is required in gamma-neurons. But it is quite confusing regarding the Dpr12-DIPdelta interactions. It is misleading to suggest that both proteins are "enriched" at the same location. I assume the authors want to propose that the projecting gamma-neurons have Dpr12 on their axons which can bind to DIPdelta (in trans, across cell membranes!), which is expressed/presented by target neurites of dopaminergic neurons.

Within the strict word limitations of the abstract, we felt that we did not have sufficient space to elaborate more on this topic. Given that, we agree with the reviewer that the Dpr-DIP interaction occurs in trans, across cell membranes, and this is reflected throughout the manuscript, including in the very next sentence in the abstract (that reads: 'DIP-δ functions in a subset of dopaminergic neurons that wire with γ-neurons within the γ4/5 zone').

Is Dpr12 really required for "developmental regrowth"? Or just for axon growth after regrowth was initiated?

We feel that this can be a philosophical question, and essentially boils down to stylistic preference. If we are forced to deliberate further on this question, Dpr12 is not required for axon growth or regrowth specifically, but for stable growth into the γ4-5 zones. In our eyes, ‘developmental regrowth’ describes the initiation, and successful completion of regrowth and circuit formation, and we therefore preferred to keep the use of this term. Moreover, in the text we refer to Dpr12/DIP-δ being required for full regrowth, or for regrowth into the γ4/5 zones.

2nd Revision - Editorial Decision 19th Feb 2021

Dear Oren,

Thank you for sending us your revised manuscript. I have now had a chance to take a careful look at it and I appreciate the introduced changes.

I am therefore very pleased to accept the revised version for publication here.

Congratulations on a nice study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

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1. Data
The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
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  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes ➔ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The differences are qualitative. We strove to image an n of at least 10 independent and representative brains in each group. n's are provided.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	not relevant as this study uses Drosophila melanogaster as a model
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	All included
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Quanticfication was performed by a double blinded ranking by an independent investigator
For animal studies, include a statement about randomization even if no randomization was used.	
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	See point 3
4.b. For animal studies, include a statement about blinding even if no blinding was done	
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Our analyses do not assume normal distribution
Is there an estimate of variation within each group of data?	This was not determined but we obtained assistance of the statistics service unit at Weizmann Institute of Science

Is the variance similar between the groups that are being statistically compared?	This was not determined but we obtained assistance of the statistics service unit at Weizmann Institute of Science

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Provided in the reagents table
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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Drosophila melanogaster, males and females, ages - throughout development from larva till one week adults
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not relevant
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Not relevant

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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The RNA-seq data has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO series accession number GEO: GSE165896 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE165896).
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