Systematic expression profiling of Dpr and DIP genes reveals cell surface codes in *Drosophila* larval motor and sensory neurons

Yupu Wang, Meike Lobb-Rabe, James Ashley, Purujit Chatterjee, Veera Anand, Hugo J. Bellen, Oguz Kanca and Robert A. Carrillo
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MS TITLE: Systematic expression profiling of *dprs* and *DIPs* reveals cell surface codes in Drosophila larval motor and sensory neurons

AUTHORS: Yupu Wang, Meike Lobb-Rabe, James Ashley, Purujit Chatterjee, Veera Anand, Hugo J Bellen, Oguz Kanca, and Robert Carrillo

Thank you for your appeal relating to your recently rejected manuscript and apologies for the long time it took me to go through the manuscript, reviews and rebuttal. My original decision was not taken without hesitation for, as you say, the referees recognise the quality of the work and it's value for future studies. However, there was some concern as to whether, without more experimental work, there was enough novel information about the function of the genes or the developmental processes they are involved in to justify publication in this journal - indeed although reviewer 3 wrote a largely positive review, they expressed uncertainty about suitability for Development in comments to the editor. I do understand the point of view you express in your rebuttal regarding the extensive time and effort that would be needed to experimentally address roles for some of the dprs/dips and you can of course make this point in your rebuttal to the reviewers. Consequently, I am happy for you to resubmit the manuscript for consideration after you address those concerns that you think are currently feasible to address. It will be important to highlight what new hypotheses are suggested by the expression analyses so that the value of the data is more evident to the readership. I will send the revised manuscript back to the reviewers explaining my decision to support submission of a revision though of course I cannot guarantee how the reviewers will respond. Upon resubmission, please provide a detailed response to the reviewers' comments and highlighting particularly any concerns that have not been included in the revised manuscript.

With best wishes,

Steve Wilson
Handling Editor
Development

Reviewer 1

Advance summary and potential significance to field

The authors have performed a massive analysis of the expression pattern of two classes of IgSF in the Drosophila nervous system. In fact, these data could be a valuable source for a further analysis of these families and their involvement in establishing connectivity pattern.

Comments for the author

Wang and co-workers have investigated the interactome in the Drosophila nervous system further. They generated a collection of GAL4 lines and UAS reporter lines to examine the expression of two extended families of the IgSF in the interactome of larval neuromuscular and sensory circuits. Interestingly, they find that each motor neuron and sensory neuron expresses a unique set of dprs and DIPs, and moreover the same classes of sensory neurons, which show similar innervation patterns, also express similar dprs and DIPs suggesting a quite complex code of these molecules for being involved in specific synaptic connectivity. Furthermore, the expression patterns are dynamic, with expression changing during development. Moreover, multiple members are expressed in gradients, furthermore suggesting a role in topographic mapping.
I think this work has been carried out very thoroughly and the gain in information is impressive. The authors outline in their Discussion various steps to move forward from these descriptive data to a functional analysis, or, in other words, to understand the logic of dprs and DIP combinatorial codes. Interestingly, the authors suggest that also target interneurons express corresponding combinations of these two families of surface molecules.
In fact, these GAL4 lines could be used as loss-of-function tools. I think for a publication in Development a few selected molecules should be analysed in this way to demonstrate their instructive involvement in formation of connectivity maps. I am fully aware that this kind of analysis will be challenging and will require a very informed selection of candidates.

Reviewer 2

Advance summary and potential significance to field

The manuscript by Wang et al catalogues the distribution of DIPs and dprs in the Drosophila larval motor and sensory neurons. The authors first generated a comprehensive set of Gal4 lines and then systematically used them to examine the expression of all known DIPs and dprs. The outcome is an extremely useful catalogue of characterized Gal4 lines that could be applied in various ways to follow and/or manipulate a particular subset of motor or sensory neurons. Furthermore, the authors used this set of reagents to describe additional motor neurons, including one innervating the alary muscle, one dedicated to muscle 23 and a pair of motor neurons that innervate m6 and m7 in the A2 segment.

These extensive analyses are carefully executed and the summaries are well-organized and relatively easy to comprehend. The community needs such a DIPs/dprs expression catalogue to make sense of the avalanche of data in the field. The authors exploited some of these new Gal4 lines to highlight specific motor neurons and to generate new “identification tags", but they stopped short from mining this rich dataset for mew insights into the DIP/dpr code and their functional interactions during neural development. This is a lost opportunity that leaves the manuscript too descriptive and without a big picture conclusion. The authors should fix this problem by re-writing the discussion. In addition, there are some presentation concerns that need to be addressed before recommending this manuscript for publication. The main issues are detailed below.

Comments for the author

1) The authors should include some examples of labeling for type II and III neurons in either the main or supplemental figures. Right now, this manuscript documents only Ib and Is terminals.
2) The summaries presented in Figs 3 and 4 are impressive. However, some concrete examples should be included in the text to orient the reader and help them appreciate the richness of the

data. For example, “we found that DIP-gamma was expressed in x1# of m15/16 (out of x2# of total observed), y1# of m30 (out of y2 observed), etc…”
3) The muscle/glia analyses (Fig 9) should be presented a lot earlier in the results section, perhaps right before the SNs paragraph entitled “Expression of dprs and DIPs in SNs”. This will help the reader asses the role of DIPs and dprs in the whole (developmental and functional) unit, that is MNs, muscles and glia together.
Also, the reader needs the muscle information to follow the paragraph entitled “Expression of dprs and DIPs is more diversified in MNs”.
4) According to Fig 9, the larval muscles express dpr10 and transiently dpr19. Does this mean that every motor neuron should express a DIP that interacts with either dpr10 or dpr19 to ensure proper target specificity? Is this true? The authors need to discuss the relevance of the muscle results relative to target recognition and synaptic connectivity.
5) It is somewhat puzzling that only one line from this large dpr/DIP-Gal4 collection (dpr1) shows expression in glia. In the discussion section the authors must further dissect this result and speculate on dpr1 function in glia and on possible interactors for dpr1 in motor neurons.
6) While I applaud the authors idea to generate unbiased hierarchical clusters, I worry that this part could generate significant confusion without some more clarifications.
First, this classification is rather coarse. The authors used the right tone in presenting and interpreting these results, but they also should discuss the caveats and limitations of such clustering methods. This will avoid misleading and instead alert the more naïve reader. Discussing the method’s caveats is particularly important for Figure 5B, where hierarchical clustering produces some unlikely correlations.
Second, the authors make little distinction between the DIPs/dprs distribution within the axonal and somato-dendritic compartments. Is it known whether DIPs/dprs expressed in a certain neuron are equally present on all neurites, that is in both axon and dendrites? This is another point that requires additional clarifications.
Finally, the hierarchical clustering of SNs and MNs should be presented in a clear parallel, with unambiguous delineation between neurons input and output and the possible role for DIPs/dprs at each end. Right now, these two parts (SNs and MNs) are difficult to follow and compare. Again, the muscle DIPs/dprs expression must be presented before this point because it needs to be discussed briefly here.
7) The discussion needs a complete reorganization.
The current discussion section lists a number of future applications but does not discuss (i) the major findings of this study, (ii) the strengths and caveats of the analyses and summaries included here, or (iii)
the implications of this work on our understanding of the DIPs and dprs.
8) In my view, this study beautifully highlights the redundancy of the DIP/dpr system throughout larval motor and sensory neurons. However, this (recurring) message is not emphasized, nor is the DIP/dpr pairing map further annotated.
The authors need to analyze and digest their data further and derive some new general observations on the role of DIPs/dprs during development. They should also use some of the examples they encountered to update the DIP/dpr cross-interaction map and to showcase the redundancy of this system.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Wang and colleagues provides a comprehensive atlas of DIP and dpr expression in motoneurons and peripheral sensory neurons. To profile DIP and dpr expression, Wang and colleagues developed a large toolbox of Gal4 strains that will undoubtedly be a useful resource for future studies. During their characterization, the authors also uncovered new insight into the innervation of several muscles, including the well-studied NMJ at m6/7 in A2.

Comments for the author

The figures are lovely and, overall, the manuscript is clearly written. The only major comment is whether this expression profile study provides new information about the logic of DIP and dpr expression that will inform our understanding of how neurons establish specific connections or

whether additional studies will be needed to test predictions based on these expression patterns. For example, does the pattern of DIP and dpr expression make sense based on the known interactions of DIPs and Dprs with each other? Some additional interpretation of the expression patterns with regards to what is known about DIP and Dpr interactions would be very useful. The authors have certainly generated a significant new library of Gal4 strains that will be a useful toolkit for functional analyses.

Additional minor comments:
1. The authors characterize the expression of DIP and dpr in sensory neurons, but of course it will be important to know what DIPs and Dprs are expressed in the ventral nerve cord, where the sensory neuron axons terminate. The motoneuron part of the story is quite well developed, since the authors profile both the motoneurons and their muscle targets, but the sensory neuron part is somewhat incomplete.
2. The expression of the Gal4 strains is sometimes difficult to discern in the merged images. It might be helpful to show the GFP (e.g. Gal4 expression) channel on its own in Figure 2B-2D.
3. The authors comment on the A-P gradient of expression of some DIPs and dprs but what might be the functional significance to this graded expression, or is it potentially just an artefact of how DIP and drp expression is regulated, as the authors suggest in the Discussion (lines 475-476)? Similar question regarding the transient expression of some of the DIPs and dprs.
4. The authors should describe the statistical analyses and tests that they ran rather than just stating that "Statistical analyses were performed using Prism 8 software" (lines 636-637).

First revision

Author response to reviewers' comments

We thank the reviewers for their insightful feedback as we think the manuscript has greatly improved. While additional data examining functional redundancy, as suggested by the *dpr/DIP* expression map, is an important next step, we think it is beyond the scope of this manuscript and would require an extensive timeline to simultaneously knockdown multiple *dprs/DIPs*. Below I discuss how we updated the revised manuscript based on the reviewers' comments. Also, please see a PDF version of these responses in the "Supplemental Information" section as formatting may change.
Comment 1 (Reviewer 1): I think for a publication in Development a few selected molecules should be analysed in this way, to demonstrate their instructive involvement in formation of connectivity maps. I am fully aware that this kind of analysis will be challenging and will require a very informed selection of candidates.
Response 1: We have analyzed single mutants for several *dprs* and *DIPs*, but we did not observe obvious connectivity phenotypes (data not shown). As suggested by the expression map, redundant and/or combinatorial Dpr/DIP codes may be required. Simultaneous knockdown of several *dprs* and *DIPs* would require extensive genetic manipulations especially since many of these genes are on the second and third chromosome. Also, although some of the GAL4s are severe hypomorphs based on mRNA abundance, many other GAL4s only modestly reduce mRNA expression. While we plan to generate null mutants for more *dprs* and *DIPs*, we think this is beyond the scope of this current manuscript.

Comment 2 (Reviewer 2): The authors exploited some of these new Gal4 lines to highlight specific motor neurons and to generate new "identification tags", but they stopped short from mining this rich dataset for mew insights into the DIP/dpr code and their functional interactions during neural development. This is a lost opportunity that leaves the manuscript too descriptive and without a big picture conclusion. The authors should fix this problem by re-writing the discussion.
Response 2: As suggested by the reviewer, we have updated the Discussion to expand upon the potential functional implications of the Dpr-DIP interactions. For example, we highlight the *dprs* expressed in muscles and their DIP interactors in motor neurons and how Dpr-DIP interactions may be part of the connectivity code (Lines 410-415). Additionally, some motor neurons co-expressed several DIPs suggesting redundant and/or combinatorial codes. In another study (Ashley et al., 2019), we examined the dorsal Is motor neuron that innervates several dorsal muscles and found

that *DIP-α* is required for specific innervation of m4 and only partially required for innervation of other muscles, suggesting that the co-expressed DIPs may be required for the additional connectivity. We further discussed this possibility in Lines 447-450.

Comment 3 (Reviewer 2): The authors should include some examples of labeling for type II and III neurons in either the main or supplemental figures. Right now, this manuscript documents only Ib and Is terminals.
Response 3: We address this comment by imaging type II and III motor neuron endings and include these new images in Figure S4, along with corresponding text (Lines 173-176).

Comment 4 (Reviewer 2): The summaries presented in Figs 3 and 4 are impressive. However, some concrete examples should be included in the text to orient the reader and help them appreciate the richness of the data. For example, "we found that DIP-gamma was expressed in x1# of m15/16 (out of x2# of total observed), y1# of m30 (out of y2 observed), etc…".
Response 4: We thank for reviewer for the suggestion and have updated the text to address this comment (Lines 171-173).

Comment 5 (Reviewer 2): The muscle/glia analyses (Fig 9) should be presented a lot earlier in the results section, perhaps right before the SNs paragraph entitled "Expression of dprs and DIPs in SNs". This will help the reader asses the role of DIPs and dprs in the whole (developmental and functional) unit, that is MNs, muscles and glia together. Also, the reader needs the muscle information to follow the paragraph entitled "Expression of dprs and DIPs is more diversified in MNs".
Response 5: The reviewer appropriately highlights that the presentation of the expression data in motor neurons, muscles, and glia should be presented in succession to better assess the role of Dprs and DIPs in the whole unit. We thank the reviewer for the suggestion and have updated the manuscript by moving the muscle and glial sections before the sensor neuron section (Lines 204-237).

Comment 6 (Reviewer 2): According to Fig 9, the larval muscles express dpr10 and transiently dpr19. Does this mean that every motor neuron should express a DIP that interacts with either dpr10 or dpr19 to ensure proper target specificity? Is this true? The authors need to discuss the relevance of the muscle results relative to target recognition and synaptic connectivity.
Response 6: We updated the Discussion section to address this comment. As pointed out by the reviewer, larval muscles express *dpr10* and *dpr19* and a majority of motor neurons express at least one DIP interactor, which could contribute to target recognition. However, some motor neurons do not express any of these DIP interactors. Thus, we do not rule out the roles for other cell surface proteins acting redundantly and/or combinatorially with Dprs and DIPs (Lines 410-415). We also provide additional testable hypotheses examining unknown interactors of Dprs and DIPs and *cis* Dpr-DIP interactions that may contribute to synaptic partner recognition (Line 415-419).

Comment 7 (Reviewer 2): It is somewhat puzzling that only one line from this large dpr/DIP-Gal4 collection (dpr1) shows expression in glia. In the discussion section the authors must further dissect this result and speculate on dpr1 function in glia and on possible interactors for dpr1 in motor neurons.
Response 7: We were not anticipating *dprs* or *DIPs* to be expressed in glia, but we were surprised to find expression of *dpr1* and no other *dprs* or *DIPs* in glia. While this data suggests that Dprs and DIPs overall do not significantly contribute to glial functions, Dpr1 has three DIP interactors which are expressed in motor neurons. Glia-motor neuron interactions are important for various aspects of motor system development, and we highlight this possibility in the Glia section of the Results (Lines 234-237).

Comment 8 (Reviewer 2): While I applaud the authors idea to generate unbiased hierarchical clusters, I worry that this part could generate significant confusion without some more clarifications. First, this classification is rather coarse. The authors used the right tone in presenting and interpreting these results, but they also should discuss the caveats and limitations of such clustering methods. This will avoid misleading and instead alert the more naïve reader. Discussing the method's caveats is particularly important for Figure 5B, where hierarchical clustering produces some unlikely correlations.

Response 8: We thank the reviewer for suggesting clarification on the cluster analyses as these interpretations must be weighed with the corresponding caveats and limitations. To address this comment, we update the Discussion alert the reader (Lines 425-429).

Comment 9 (Reviewer 2): The authors make little distinction between the DIPs/dprs distribution within the axonal and somato-dendritic compartments. Is it known whether DIPs/dprs expressed in a certain neuron are equally present on all neurites, that is in both axon and dendrites? This is another point that requires additional clarifications.
Response 9: The distribution of Dprs and DIPs to specific pre- and postsynaptic compartments will impact interactions between cognate pairs and contribute to their function. In a previous study, we observed DIP-α localization to motor neuron axons (Ashley et al., 2019). Other labs, including the Zipursky lab, showed that DIP-α, Dpr6, and Dp10 are found in specific layers in optic lobe neuropils although axons and dendrites were not distinguished (Xu et al., 2018). We updated the Discussion to highlight the importance of examining the distribution of Dprs and DIPs (Lines 517-520).

Comment 10 (Reviewer 2): The hierarchical clustering of SNs and MNs should be presented in a clear parallel, with unambiguous delineation between neurons input and output and the possible role for DIPs/dprs at each end. Right now, these two parts (SNs and MNs) are difficult to follow and compare. Again, the muscle DIPs/dprs expression must be presented before this point because it needs to be discussed briefly here.
Response 10: The clustering analyses of motor neurons and sensory neurons were purposefully presented after the sensory neuron expression to facilitate interpretation and presentation of the clustering methodology and results as they were generated by the same pipeline. Nonetheless, we reorganized the *dpr/DIP* expression sections and now, the muscle section immediately follows the motor neuron section to better appreciate the data.

Comment 11 (Reviewer 2): The current discussion section lists a number of future applications but does not discuss (i) the major findings of this study, (ii) the strengths and caveats of the analyses and summaries included here, or (iii) the implications of this work on our understanding of the DIPs and dprs.
Response 11: We thank the reviewer for the suggestions to bolster the Discussion. The major findings of the study are briefly summarized in Lines 397-404, and we expand the implications of this study throughout the Discussion and in the Result section as well. For example, we now highlight specific Dpr-DIP interactors that may contribute to motor neuron-muscle recognition (Lines 410-415) and glia-motor neuron interactions (Lines 234-237), and we now discuss how co-expressed DIPs may act redundantly and/or combinatorially to wire specific motor neuron branches (Lines 447-450). To discuss the strengths and caveats, we include a new section in the Discussion "Limitations of using GAL4 lines to profile expression patterns" (Lines 515-526).

Comment 12 (Reviewer 2): In my view, this study beautifully highlights the redundancy of the DIP/dpr system throughout larval motor and sensory neurons. However, this (recurring) message is not emphasized, nor is the DIP/dpr pairing map further annotated.

Response 12: We agree that redundancy likely plays a significant role in Dpr/DIP functions, and we highlight not only the potential redundancy in Dprs and DIPs but in other cell surface proteins as well that are implicated in synaptic partner recognition (Lines 439-450).

Comment 13 (Reviewer 2): The authors need to analyze and digest their data further and derive some new general observations on the role of DIPs/dprs during development. They should also use some of the examples they encountered to update the DIP/dpr cross-interaction map and to showcase the redundancy of this system.
Response 13: We have included additional hypotheses for Dprs and DIPs during nervous system development based on the expression analyses. However, as discussed in Response 1, examining the functional redundancy is beyond the scope of this study and would require significant time and resource investment. These experiments are part of our longer-term goals and will be included in future work.

Comment 14 (Reviewer 3): Does the pattern of DIP and dpr expression make sense based on the known interactions of DIPs and Dprs with each other? Some additional interpretation of the

expression patterns with regards to what is known about DIP and Dpr interactions would be very useful.
Response 14: Similar to our responses to Comments 2, 6, 7, 11, and 12, we delve deeper into how the expression patterns of Dpr-DIP interactors may instruct specific motor neuron-muscle and glia-motor neuron interactions. The roles of these interactions will be an exciting avenue for future experiments.

Comment 15 (Reviewer 3): The authors characterize the expression of DIP and dpr in sensory neurons, but of course it will be important to know what DIPs and Dprs are expressed in the ventral nerve cord, where the sensory neuron axons terminate. The motoneuron part of the story is quite well developed, since the authors profile both the motoneurons and their muscle targets, but the sensory neuron part is somewhat incomplete.
Response 15: Revealing the expression patterns of *dprs* and *DIPs* in interneurons will certainly inform which Dpr-DIP interactions may instruct sensory neuron-interneuron recognition. Because the ventral nerve cord is densely packed, we cannot discern specific interneurons using our current experimental pipeline. In future work we will focus on specific sensory neurons with identified interneuron partners and determine the expression of *dprs* and *DIPs* within those cells by co-labeling with specific transcription factors. We discussed this limitation in Line 520-523.

Comment 16 (Reviewer 3): The expression of the Gal4 strains is sometimes difficult to discern in the merged images. It might be helpful to show the GFP (e.g. Gal4 expression) channel on its own in Figure 2B-2D.
Response 16: We thank the reviewer for pointing this out and have updated Figure 2.

Comment 17 (Reviewer 3): The authors comment on the A-P gradient of expression of some DIPs and dprs, but what might be the functional significance to this graded expression, or is it potentially just an artefact of how DIP and drp expression is regulated, as the authors suggest in the Discussion (lines 475-476)? Similar question regarding the transient expression of some of the DIPs and dprs.
Response 17: The A-P gradient and transient expression patterns of some *dprs* and *DIPs* may provide important clues to their function. (1) To date, we do not understand how gradient transcriptional factors regulate segmental development in the nervous system. We agree with the reviewer that the gradient expression of *dprs* and *DIPs* may be artefact, but these genes also serve as great candidates to set up segmental cues due to the similar expression patterns. (2) In addition, a small subset of *dprs* and *DIPs* are transiently expressed, suggesting that Dprs and DIPs are multifunctional. Indeed, Dpr-DIP interactions are implicated in early (e.g. synaptic partner recognition) and late (e.g. NMJ morphology) steps of circuit development. We updated the manuscript and highlight these possibilities in Lines 191-192 and 472-475.

Comment 18 (Reviewer 3): The authors should describe the statistical analyses and tests that they ran rather than just stating that "Statistical analyses were performed using Prism 8 software" (lines 636-637).
Response 18: We thank the reviewer for pointing out this oversight on our part and updated the corresponding Methods sections (Lines 668-669).

We hope that the updated manuscript is now satisfactory for the reviewers and *Development* editors. Thank you in advance and we look forward to hearing from you.

Second decision letter

MS ID#: DEVELOP/2021/200355

MS TITLE: Systematic expression profiling of *dprs* and *DIPs* reveals cell surface codes in *Drosophila* larval motor and sensory neurons

AUTHORS: Yupu Wang, Meike Lobb-Rabe, James Ashley, Purujit Chatterjee, Veera Anand, Hugo J Bellen, Oguz Kanca, and Robert Carrillo
ARTICLE TYPE: Research Article
I am happy to tell you that the reviewers are happy with your revisions and your manuscript has been accepted for publication in Development, pending our standard ethics checks. The referee reports are appended below.

Reviewer 1

Advance summary and potential significance to field

Wang and co-workers have investigated the interactome in the Drosophila nervous system further. They generated a collection of GAL4 lines and UAS reporter lines to examine the expression of two extended families of the IgSF in the interactome of larval neuromuscular and sensory circuits.

I think this work has been carried out very thoroughly and the gain in information is impressive. The authors outline in their Discussion various steps to move forward from these descriptive data to a functional analysis, or, in other words, to understand the logic of dprs and DIP combinatorial codes.
Interestingly, the authors suggest that also target interneurons express corresponding combinations of these two families of surface molecules.

Comments for the author

I do acknowledge the arguments of the authors that they tried hard (but unsuccessfully) to obtain connectivity phenotypes after knockdown of single candidate molecules. In fact, the next step to knockout a combination of candidate molecules might be very work-intensive and possibly beyond the scope of this initial paper.
I do acknowledge also the insightful comments of reviewers 2 and 3 which were more in support of the main conclusions of the paper, and the thorough response of the authors which indeed has improved the quality of the manuscript substantially.

Reviewer 2

Advance summary and potential significance to field

In the revised manuscript the authors have addressed all the comments and concerns that I raised before.

Comments for the author

In the revised manuscript the authors have addressed all the comments and concerns that I raised before.
Congratulations to the authors for a very nice piece of work.

Reviewer 3

Advance summary and potential significance to field

This ms. provides a clear, comprehensive analysis of the expression patterns of a family of cell-surface proteins, DIPs/dprs, in the context of the developing nervous system, specifically the neuromuscular junction. The study has generated a suite of reagents for future function-probing analysis of the potential logic of DIP/dpr expression patterns.

Comments for the author

The authors have nicely revised their manuscript in response to the reviewers' comments. There is still the concern that the manuscript is descriptive, but the authors present a nice body of work.