nature portfolio

Peer Review File

# Conserved autism-associated genes tune social feeding behavior in C. elegans

Corresponding Author: Mr Michael Hart

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

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Cowen et al use aggregation behavior in C. elegans as a vehicle to study how neurexin, neuroligin and other autism-related genes contribute to circuit function and behavior. The authors take advantage of previous work that identified a set of interconnected neurons, including the URX oxygen sensors, the ASH and ADL nociceptors, and the RMG interneurons, that promotes and regulates C. elegans aggregation. The authors show that disrupting the worm orthologs of neurexin, nrx-1, or of neuroligin, nlg-1, reduces aggregation. They find that NRX-1 acts in the ASH and ADL neurons to promote aggregation, and that it does so independently of NLG-1 They find moreover that glutamate release from ASH and ADL promotes aggregation, acting through a set of glutamate receptors that include GLR-1, GLR-2 and AVR-15. They also show that social animals display faster presynaptic release from ASH neurons than solitary animals, and have a larger number of presynaptic release sites in these neurons. Using compound mutations and behavioral analyses they show that the pathways they identify act at least partly independently.

The data are clearly presented and straightforward to interpret. However, I have some suggestions for improving the manuscript, which I list below.

Main comments
1) The authors report that social animals have more CLA-1::GFP puncta in ASH neurons than solitary animals. This implies that signalling from the NPR-1 neuropeptide receptor influences the synaptic anatomy of these neurons, an interesting finding. Previous work suggests that NPR-1 acts primarily in the RMG neurons to regulate aggregation behavior. The authors should examine if expressing NPR-1 in RMG neurons is sufficient to rescue the ASH synaptic phenotypes (implicating a cell non-autonomous mechanism), or whether NPR-1 functions cell autonomously in ASH to regulate CLA-1::GFP puncta numbers.

2) The authors also report that in social animals ASH neurons exhibit faster/increased release of neurotransmitter compared to solitary animals. Again, the authors should examine if this altered release reflects cell-autonomous effects of NPR-1 in ASH, or non-cell-autonomous effects in RMG.

3) The authors implicate NLG-1 in regulating aggregation behavior, but attempts at establishing where NLG-1 is required in the circuit, using targeted expression to rescue the aggregation defect, were unsuccessful. This may mean that NLG-1 is required in multiple neurons to promote aggregation. I suggest the authors employ the reverse strategy to tackle this important question, using targeted auxin-inducible degradation of NLG-1. By this I mean knocking in a degron to the C-terminal cytoplasmic tail of NLG-1 and degrading it specifically in ASH, ADL, RMG and URX by targeted TIR1 expressiom, and looking for aggregation phenotypes. This may provide insights into different functions of NLG-1 in different neurons, an important focus of the study.

4) In Figure 2 the authors use the nhr-79 promoter and a combination of the srv-3 and sra-6 promoters to provide compelling evidence that NRX-1acts in ASH and ADL neurons to promote aggregation. Using two different promoters helps reduce the likelihood that rescue is due to low expression in a different neuron that may not be detected by examining GFP expression from the transgene. It would be relatively easy to confirm that not only the nhr-79 promoter but also a combining the srv-3p::eat-4 and sra-6p::eat-4 transgenes rescues the eat-4 phenotype.

5) In the discussion (Pg 23 – 24), the authors go to some length to explain that C. elegans aggregation does not provide a model for autism, but rather provides 'a framework to explore the molecular functions of autism-associated genes in social behaviors in more complex model systems'. I suggest the authors move this explicit statement to the introduction. This avoids any misunderstanding that they are studying social / solitary foraging behavior in C. elegans because it is evolutionarily related to variation in social behaviors in mammals.

Minor comments
1) Pg 2 L16 'npr-1 (NPY1R)' change to 'npr-1 (neuropeptide receptor 1)'
2) Pg 2 L21 'npr-1 modifies behavior through inhibition of RMG interneurons.'
It would be more accurate to say 'npr-1 acts in RMG interneurons to inhibit aggregation behavior.'
3) Pg 4 L12 'Through mechanistic study, we also identify….highlighting conservation of this pathway across species.' This sentence can be misleading, as it suggests an evolutionary relationship between autism and C. elegans aggregation, which is not supported by the evidence.

Reviewer #2

(Remarks to the Author)
The manuscript by Cowen and colleagues demonstrated three independent molecularly synaptic mechanisms that turn foraging behavior in C. elegans from solitary to social. These molecules include the presynaptic adhesion molecule NRXN-1 and its canonical postsynaptic binding partner, NLG-1, but work independently in aggregation behavior. Although synaptic glutamate transmission regulates aggregate feeding in an NRXN-1-independent manner, nrxn-1 modulates presynaptic architecture (monitored via presynaptic puncta) in social feeding. Besides, aggregate feeding induced by circuit activation also requires nrxn-1.

The overall finding of autism-associated genes, nrxns and nlgs, in synaptic mechanisms underlying social foraging behavior is compelling. The experiments are well-designed, and the manuscript is well-written. A few points should be added to improve the concepts of synaptic adhesion molecules in regulating synapses and synaptic transmission contributing to social feeding.

1. Although NRXNs have been indicated to function in the distribution of synaptic glutamate receptors, NLGs structurally allocate glutamate receptors at postsynaptic terminal. Fig. 4D determined additional effects in loss of glutamate signaling and NLG-1 that drove the feeding behavior close to solitary control, suggesting the parallel pathways of glutamate and NLG-1 in aggregation behavior. However, it is required to examine in Fig. 4E whether the role of NLG-1 in social feeding occludes that of glutamate receptors before concluding whether glutamate affects feeding behavior independently of NLG-1.

2. Higher glutamate release was coupled with aggregate feeding in Fig. 5D. It is puzzling that npr-1;nrxn-1 significantly reduced social feeding; however, the levels of glutamate release in npr-1 and npr-1;nrxn-1 were indistinguishable in Fig. 5D. Although the phenotype of npr-1;nrxn-1 in feeding behavior could be resulted from the reduction of presynaptic releasing site as demonstrated in Fig 6D, will expression of NRXN-1 in npr-1;nrxn-1 change glutamate release or restore the augment of presynaptic puncta induced by npr-1?

3. It is appreciable that Fig. 7 exhibited the role of nrxn-1 in aggregation behavior independent of npr-1. To strengthen the function of nrxns in social feeding behavior, whether simply expressing NRXN-1 can boost aggregate feeding in solitary control?

4. There is a minor point regarding the resolution of Fig 5A.

Reviewer #3

(Remarks to the Author)
Cowen, Hart and colleagues report the effects of neurexin and neuroligin mutation on a circuit that control aggregation behavior of C. elegans. They find genetically separable roles for the neurexin homolog nrx-1 and the neuroligin homolog nlg-1 as modifiers of aggregation caused by a loss-of-function mutation in the neuropeptide receptor gene npr-1. They find that ADL and ASH sensory neurons are a site of action for nrx-1, and that nrx-1 and nlg-1 function in parallel to glutamatergic neurotransmission. Although there is evidence of increased vesicle fusion in sensory neurons of npr-1 (aggregating) mutants, nrx-1 mutation does not affect this aspect of neuronal function. Finally, the authors report that nrx-1 mutation affects the number of presynaptic zones in ASH sensory neurons.

This study provides clear evidence that neurexin and neuroligin homologs function in a neural circuit that controls aggregation of C. elegans, which can be considered a model for social behavior. The study has several strengths. First, the investigators use diverse approaches to measure the effects of nrx-1/nlg-1 mutation on this circuit to reveal that these genes have separable functions. The experiments are well controlled and, for the most part, clearly explained and presented. Neurexin and neuroligin are important risk factors for neurodevelopmental disorders and this study describes new opportunities to use model organism genetics to study their function.

The study also has several weaknesses. The site of nlg-1 action remains unresolved making it unclear how it functions in relation to nrx-1. Although the authors measure effects of nrx-1 mutation on synapse number, this has no measurable impact

on presynaptic function making it difficult to understand how nrx-1 functions in relation to synaptic glutamate signaling. Throughout the manuscript, data are replotted without being clearly labeled as such, and it isn't clear that statistical analysis take this into account. And the study ends with an experiment showing that an engineered gain-of-function in protein kinase C also causes an aggregation phenotype that can be modified by nrx-1 mutation - it is not clear what this adds to the story and why it is a logical stop-point for the study.

Specific comments follow.

1. nrx-1 site of action is defined using flp-21 and nhr-79 promoters. nlg-1 site-of-action studies use a different promoter and report no effect. It is, perhaps, possible that if the investigators used the same promoters for both experiments they might find a site of action for nlg-1. They should also be cautious interpreting the absence of rescue using nlg-1 transgenes, especially as their positive control shows only partial rescue.
2. Figure 2D needs a label clearly indicating that the figure shows GFP::NRX-1 fluorescence.
3. Throughout the study please clearly indicate when data are replotted. Please make clear in the methods section that appropriate corrections for multiple comparisons have been used.
4. The authors' model predicts that mutation of glutamate receptors would have no effect in the absence of glutamatergic neurotransmission i.e. an eat-4 mutant. The authors should test this.
5. The authors refer to avr-15 as a glycine receptor homolog, which might be confusing as it is a glutamate-gated chloride channel. Also, it is not clear whether avr-15 is more similar to glycine or GABA receptors. If the authors wish to make the point that AVR-15 channels are possible GlyR homologs, it should be explained more clearly and better supported.
6. The authors equate pHluorin recovery with glutamate release - this is not necessarily the case - this is an exocytosis assay and reflects the amount of membrane fusion.
7. Figure 5 is pixellated. Also, the cartoon depicting VGLUT::pHluorin makes VGLUT look like a channel, which might be confusing.
8. Why was ADL ignored in pHluorin recovery experiments?
9. It isn't clear whether Figure 5D shows summary data from all experiments or representative data from one trial. Summary data of all the recovery slopes from all the trials should be shown in the main figures as scatter plots.
10. The investigators measure effects of nrx-1 mutation on the number of CLA-1 puncta. What about size of puncta? Representative micrographs leave open the possibility that the size of puncta is affected.
11. Why is CLA-1 signal affected by nrx-1 mutation but basal levels of EAT-4::pHluorin not? These are both presynaptic markers.
12. What do asterisks in Figure 6E mean?
13. Figure 7 - these data seem to better fit in the earlier part of the narrative when the effect of nrx-1 mutation on aggregation is first reported. Also, it isn't clear that the effect of pkc-1(gf) will be modified by all the perturbations that modify npr-1(lf).
14. The discussion point about an ancestral role for these genes in social behavior might be a bridge too far.

Minor comments:
p. 10 line 5 reword - these data are consistent with prior observations
p. 11 line 23 reword 'trans-spliced GFP' should refer to coding sequences
p. 28 line 22 typo 'blech' should be 'bleach'

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have performed most of the experiments I suggested. While the data they obtain from them do not neatly tie up the loose ends of the story, they are thought-provoking. They suggest considerable complexity in how this circuit functions.

Reviewer #2

(Remarks to the Author)
The authors already addressed my comments. I don't have further concerns.

Reviewer #3

(Remarks to the Author)
The revised manuscript by Cowen and colleagues has been strengthened with additional data and by altering the structure of the narrative and edits to the text. My major comments have been addressed. I have notes on minor corrections that the authors can make.

(1) Page 4 lines 12-14 is awkwardly written and could confuse the reader.

(2) Page 5 line 9 - the authors could convert the number of animals interacting into a fraction, i.e. 39 animals (out of 50) would instead be 78%.

(3) Page 6 line 7 - the authors refer to expression of pkc-1(gf) in neurons that provide peptidergic input to RMGs as 'outside

the context of npr-1.' This is confusing. The experiment can (and probably should) be interpreted as indicating that gain-of-function in the NPR-1 signaling pathway causes similar effects regardless of whether RMGs or cells that regulate RMGs are targeted.

(4) Please check Results section for consistent use of the past tense.

(5) Please check figure legends for consistent use of 'data are' instead of 'data is.'

(6) The authors tend to over-hedge some statements, especially in the results. For example, on page 8 lines 15-18 the authors write that 'finding suggest' that genes 'likely function in parallel.' It suffices to write that the findings suggest parallel action. Please check the text for other instances of this over-hedging.

(7) Page 13 line 3 - the description of CLA-1 imaging is confusing. First, the authors refer to the method used as 'enhanced resolution confocal microscopy.' What does this mean? Second, the authors write that they image a 'chemical GFP-tagged pre-synaptic marker.' What does this mean? My understanding was that these experiments involved conventional imaging of GFP-tagged CLA-1. Did I miss something?

(8) I encourage the authors to use more of the Discussion section to synthesize their observations into models and speculate. As written, the Discussion section devotes a little too much bandwidth to summarizing the study.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
The authors have addressed all my comments.

Please find below the response to each of the reviewer's comments.

Reviewer #1 (Remarks to the Author):

Cowen et al use aggregation behavior in C. elegans as a vehicle to study how neurexin, neuroligin and other autism-related genes contribute to circuit function and behavior. The authors take advantage of previous work that identified a set of interconnected neurons, including the URX oxygen sensors, the ASH and ADL nociceptors, and the RMG interneurons, that promotes and regulates C. elegans aggregation. The authors show that disrupting the worm orthologs of neurexin, nrx-1, or of neuroligin, nlg-1, reduces aggregation. They find that NRX-1 acts in the ASH and ADL neurons to promote aggregation, and that it does so independently of NLG-1 They find moreover that glutamate release from ASH and ADL promotes aggregation, acting through a set of glutamate receptors that include GLR-1, GLR-2 and AVR-15. They also show that social animals display faster presynaptic release from ASH neurons than solitary animals, and have a larger number of presynaptic release sites in these neurons. Using compound mutations and behavioral analyses they show that the pathways they identify act at least partly independently.

The data are clearly presented and straightforward to interpret. However, I have some suggestions for improving the manuscript, which I list below.

We thank the reviewer for their positive comments.

Main comments
1) The authors report that social animals have more CLA-1::GFP puncta in ASH neurons than solitary animals. This implies that signalling from the NPR-1 neuropeptide receptor influences the synaptic anatomy of these neurons, an interesting finding. Previous work suggests that NPR-1 acts primarily in the RMG neurons to regulate aggregation behavior. The authors should examine if expressing NPR-1 in RMG neurons is sufficient to rescue the ASH synaptic phenotypes (implicating a cell non-autonomous mechanism), or whether NPR-1 functions cell autonomously in ASH to regulate CLA-1::GFP puncta numbers.

We agree with the reviewer that is an interesting finding, and now include a comparison of ASH::gfp::cla-1 puncta between animals with and without NPR-1 in RMG (*nlp-56p::npr-1::SL2::mCherry*) and ASH (*sra-6p::npr-1::SL2::mCherry*). Neither transgene impacts ASH::cla-1 puncta, suggesting NPR-1 is likely not acting in a cell autonomous manner in ASH, or alone in RMG. This analysis is now included in Figure 6 and in the text:
"The difference in ASH presynaptic architecture between solitary controls and aggregating *npr-1(ad609)* animals could be due to *npr-1* functioning broadly to alter activity of the circuit or in a cell autonomous manner. Expression of *npr-1* in ASH (*sra-6p::npr-1*) or RMG (*nlp-56p::npr-1*) neurons in *npr-1(ad609)* animals did not impact ASH CLA-1::GFP puncta (**Fig. 6F&G**), which suggests *npr-1* is required in other neurons or more broadly in the circuit to impact ASH synaptic numbers." – Page 13 lines 22-23, Page 14 lines 1-4

2) The authors also report that in social animals ASH neurons exhibit faster/increased release of neurotransmitter compared to solitary animals. Again, the authors should examine if this altered release reflects cell-autonomous effects of NPR-1 in ASH, or non-cell-autonomous effects in RMG.

We agree with the reviewer that this is an interesting experiment. However, given the lack of impact with npr-1 transgenes on ASH puncta mentioned above, and the relatively small difference we observe the glutamate release FRAP studies, we do not think we would observe meaningful changes with the npr-1 transgenes. Moreover, nrx-1 is not required for this change in the rate of neurotransmitter release, so we believe this experiment would not impact the major conclusions of the manuscript.

3) The authors implicate NLG-1 in regulating aggregation behavior, but attempts at establishing where NLG-1 is required in the circuit, using targeted expression to rescue the aggregation defect, were unsuccessful. This may mean that NLG-1 is required in multiple neurons to promote aggregation. I suggest the authors employ the reverse strategy to tackle this important question, using targeted auxin-inducible degradation of NLG-1. By this I mean knocking in a degron to the C-terminal cytoplasmic tail of NLG-1 and degrading it specifically in ASH, ADL, RMG and URX by targeted TIR1 expression, and looking for aggregation phenotypes. This may provide insights into different functions of NLG-1 in different neurons, an important focus of the study.

We agree with the reviewer that this approach would be powerful in determining the location of action of nlg-1. To address this concern, we expanded the panel of neurons/tissues using transgenes to restore expression of nlg-1 using its own promoter (*nlg-1p::nlg-1::gfp*), a muscle promoter (*myo-3p::nlg-1*), and a promoter for the interneuron AIY (*ttx-3p::nlg-1*). The integrated *nlg-1p* transgene did not rescue behavior, but these animals appear sluggish and slow growing so we think there are issues with overexpression of NLG-1 that may impact behavior. We also found no change in behavior with muscle expression but did observe an increase in aggregation with expression of nlg-1 in AIY interneurons. This analysis is included in Figure 3 and Supplemental Figure 2, and in the text:

"Expression of sfGFP::NLG-1 in ADL and ASH sensory neurons (*nhr-79* promoter), in the RMG interneurons (*nlp-56* promoter), in the body wall muscles (*myo-3* promoter), or expression of GFP::NLG-1 with the *nlg-1* promoter (integrated transgene) did not impact aggregation behavior (**Fig. 3C**). Expressing sfGFP::NLG-1 in ADL (*srv-3* promoter) or ASH (*sra-6* promoter) individually or in AIA (*ins-1* promoter) did not rescue aggregation behavior (**Supplemental Fig. 2A**), but expression of sfGFP::NLG-1 in AIY neurons did increase the number of aggregating animals similar to levels with expression in all neurons (**Fig. 3C**). We also confirmed expression of all sfGFP::NLG-1 transgenes by analyzing expression by the sfGFP or GFP tag (**Supplemental Fig. 2B**). Together, these results imply that NLG-1 in neurons or in the AIY neurons is sufficient to partially modify aggregation behavior. The *nlg-1p* transgene surprisingly did not impact behavior but may be overexpressed at a level that

interferes with the behavior as these animals were also sluggish and somewhat slow growing." – Page 8, lines 1-12.

4) In Figure 2 the authors use the nhr-79 promoter and a combination of the srv-3 and sra-6 promoters to provide compelling evidence that NRX-1acts in ASH and ADL neurons to promote aggregation. Using two different promoters helps reduce the likelihood that rescue is due to low expression in a different neuron that may not be detected by examining GFP expression from the transgene. It would be relatively easy to confirm that not only the nhr-79 promoter but also a combining the srv-3p::eat-4 and sra-6p::eat-4 transgenes rescues the eat-4 phenotype.

We were able to combine the srv-3p::eat-4 and sra-6p::eat-4 transgenes as suggested by the reviewer and find this increases the number of aggregating animals as expected (new data included in Figure 4. Combination of the original transgenes together did not alter behavior, but by increasing the injection concentration and expression of sra-6p::eat-4, we observed an increase in aggregation behavior with combination of the transgenes (the higher concentration sra-6p::eat-4 transgene did not impact behavior alone). This is included in Figure 4 and in the text:
"To further explore the interplay of *nrx-1* and glutamate in ADL and ASH sensory neurons, we expressed EAT-4 or NRX-1(α) in these neurons of *npr-1(ad609)*; *nrx-1(wy778)*; *eat-4(ky5)* triple mutants using the *nhr-79* promoter or the *srv-3* and *sra-6* promoters combined. Expression of EAT-4 in ADL and ASH with *nhr-79p* or combination of *srv-3* and *sra-6* in the *npr-1(ad609)*; *nrx-1(wy778)*; *eat-4(ky5)* triple mutants restored aggregation behavior to the level of *npr-1(ad609);nrx-1(ad609)* (**Fig. 4B**)" -- Page 10, lines 7-12

5) In the discussion (Pg 23 – 24), the authors go to some length to explain that C. elegans aggregation does not provide a model for autism, but rather provides 'a framework to explore the molecular functions of autism-associated genes in social behaviors in more complex model systems'. I suggest the authors move this explicit statement to the introduction. This avoids any misunderstanding that they are studying social / solitary foraging behavior in C. elegans because it is evolutionarily related to variation in social behaviors in mammals.

We agree and have now added this point in the introduction text: "These additive neuronal mechanisms exemplify the complexity of *C. elegans* foraging strategies and although it is not a model for autism, it provides insights and a framework for how variation in social behavior is achieved at the genetic, molecular, and circuit levels, which may be applied to more complex organisms." – Page 4, lines 15-19

Minor comments –
1) Pg 2 L16 'npr-1 (NPY1R)' change to 'npr-1 (neuropeptide receptor 1)' – we have added *npr-1*(ortholog to human NPY1R) – Page2, line16
2) Pg 2 L21 'npr-1 modifies behavior through inhibition of RMG interneurons.'
It would be more accurate to say 'npr-1 acts in RMG interneurons to inhibit aggregation

behavior.' - – we have edited to incorporate this "*npr-1* inhibits aggregation behavior acting in RMG interneurons" – Page 2, lines 21- 22

3) Pg 4 L12 'Through mechanistic study, we also identify….highlighting conservation of this pathway across species.' This sentence can be misleading, as it suggests an evolutionary relationship between autism and C. elegans aggregation, which is not supported by the evidence.

We have removed the last part of this sentence to avoid this confusion – "We also use genetic methods to identify the downstream glutamate receptors that regulate aggregation behavior, homologs of which are also associated with autism." – Page 4, lines 10-11

Reviewer #2 (Remarks to the Author):

The manuscript by Cowen and colleagues demonstrated three independent molecularly synaptic mechanisms that turn foraging behavior in C. elegans from solitary to social. These molecules include the presynaptic adhesion molecule NRXN-1 and its canonical postsynaptic binding partner, NLG-1, but work independently in aggregation behavior. Although synaptic glutamate transmission regulates aggregate feeding in an NRXN-1-independent manner, nrxn-1 modulates presynaptic architecture (monitored via presynaptic puncta) in social feeding. Besides, aggregate feeding induced by circuit activation also requires nrxn-1.

The overall finding of autism-associated genes, nrxns and nlgs, in synaptic mechanisms underlying social foraging behavior is compelling. The experiments are well-designed, and the manuscript is well-written. A few points should be added to improve the concepts of synaptic adhesion molecules in regulating synapses and synaptic transmission contributing to social feeding.

1. Although NRXNs have been indicated to function in the distribution of synaptic glutamate receptors, NLGs structurally allocate glutamate receptors at postsynaptic terminal. Fig. 4D determined additional effects in loss of glutamate signaling and NLG-1 that drove the feeding behavior close to solitary control, suggesting the parallel pathways of glutamate and NLG-1 in aggregation behavior. However, it is required to examine in Fig. 4E whether the role of NLG-1 in social feeding occludes that of glutamate receptors before concluding whether glutamate affects feeding behavior independently of NLG-1.

As we identified multiple glutamate receptors that are involved in aggregation behavior, to truly dissect the potential unexpected interaction suggested by the reviewer would require combining mutations of nlg-1 with all three receptors, and in multiple combinations. While this would be interesting, we believe it is beyond the scope of this manuscript. To address this concern we generated a strain mutant for both nlg-1 and one of the glutamate receptors (glr-2). This is included in Figure 4 and the results text:

"Surprisingly, we find that the reduced aggregation phenotype of *nlg-1* is dependent on *glr-2*, as the *nlg-1; glr-2* combination increased aggregation compared to *nlg-1* alone, to levels similar to *glr-2* alone (**Fig. 4D**). Therefore *nlg-1* and *nrx-1* have distinct genetic interactions with *glr-2*." – Page 11, lines 16-19

And in the discussion text:
"*nlg-1* and *nrx-1* show opposite genetic interactions with *glr-2*, where the *nlg-1* aggregation phenotype reverts to the lesser *glr-2* phenotype when combined, which although surprising, is similar to an interaction we recently observed between *nrx-1* and *nlg-1*[89]." – Page 15, lines 15-18

2. Higher glutamate release was coupled with aggregate feeding in Fig. 5D. It is puzzling that npr-1;nrxn-1 significantly reduced social feeding; however, the levels of glutamate release in npr-1 and npr-1;nrxn-1 were indistinguishable in Fig. 5D. Although the phenotype of npr-1;nrxn-1 in feeding behavior could be resulted from the reduction of presynaptic releasing site as demonstrated in Fig 6D, will expression of NRXN-1 in npr-1;nrxn-1 change glutamate release or restore the augment of presynaptic puncta induced by npr-1?

As mentioned above in response to reviewer 1, we are concerned that the relatively small difference we observe in FRAP experiments would make it difficult to test transgene rescue with available methods. Further, we did not observe a cell autonomous impact on ASH::cla-1 puncta using npr-1 transgenes, which is the more relevant experiment regarding cell autonomy and the main focus of our analysis of the nrx-1 locus.

3. It is appreciable that Fig. 7 exhibited the role of nrxn-1 in aggregation behavior independent of npr-1. To strengthen the function of nrxns in social feeding behavior, whether simply expressing NRXN-1 can boost aggregate feeding in solitary control?

We include analysis of aggregation behavior with expression of NRX-1 in all neurons in solitary control animals and find no increase in aggregation behavior. These results are included in Supplemental Figure 1 and the text:
"Neuronal expression of NRX-1 in solitary controls, which should result in overexpression of the α-isoform, did not increase aggregation behavior (**Supplemental Fig. 1E**)." – Page 6, line 23, Page 7, line 1.

4. There is a minor point regarding the resolution of Fig 5A.

We have ensured this figure is now at the same resolution as the other figures.

Reviewer #3 (Remarks to the Author):

Cowen, Hart and colleagues report the effects of neurexin and neuroligin mutation on a circuit that control aggregation behavior of C. elegans. They find genetically separable roles for the neurexin homolog nrx-1 and the neuroligin homolog nlg-1 as modifiers of aggregation caused by a loss-of-function mutation in the neuropeptide receptor gene npr-1. They find that ADL and ASH sensory neurons are a site of action for nrx-1, and that nrx-1 and nlg-1 function in parallel to glutamatergic neurotransmission. Although there is evidence of increased vesicle fusion in sensory neurons of npr-1 (aggregating) mutants, nrx-1 mutation does not affect this aspect of neuronal function. Finally, the authors report that nrx-1 mutation affects the number of presynaptic zones in ASH sensory neurons.

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The study also has several weaknesses. The site of nlg-1 action remains unresolved making it unclear how it functions in relation to nrx-1. Although the authors measure effects of nrx-1 mutation on synapse number, this has no measurable impact on presynaptic function making it difficult to understand how nrx-1 functions in relation to synaptic glutamate signaling. Throughout the manuscript, data are replotted without being clearly labeled as such, and it isn't clear that statistical analysis take this into account. And the study ends with an experiment showing that an engineered gain-of-function in protein kinase C also causes an aggregation phenotype that can be modified by nrx-1 mutation - it is not clear what this adds to the story and why it is a logical stop-point for the study.

We thank the reviewer for helpful comments and experimental ideas. For the protein kinase C experiment, we have kept this in the manuscript as it was appreciated by reviewer 2, but have moved it to Figure 1 and Supplemental Figure 1 to support those conclusions and not be the stop-point for the study.

Specific comments follow.

1. nrx-1 site of action is defined using flp-21 and nhr-79 promoters. nlg-1 site-of-action studies use a different promoter and report no effect. It is, perhaps, possible that if the investigators used the same promoters for both experiments they might find a site of action for nlg-1. They should also be cautious interpreting the absence of rescue using nlg-1 transgenes, especially as their positive control shows only partial rescue.

We appreciate this comment and agree with the caveats mentioned by the reviewer. In order to address this concern, we expanded the panel of neurons/tissues using transgenes to restore expression of nlg-1 using its own promoter (*nlg-1p::nlg-1::gfp*), a muscle promoter (myo-3p::nlg-1), and a promoter for the interneuron AIY (ttx-3p). We found that the integrated transgene did not rescue behavior, which may be due to overexpression of NLG-1 (these animals appear sluggish and slow growing). We also found no change in behavior with muscle expression alone but did observe an increase with expression of nlg-1 in AIY interneurons similar to that observed with expression in all neurons (ric-19p). This is now included in Figure 3 and Supplemental Figure 2 and in the text:
"Expression of sfGFP::NLG-1 in ADL and ASH sensory neurons (*nhr-79* promoter), in the RMG interneurons (*nlp-56* promoter), in the body wall muscles (*myo-3* promoter), or expression of GFP::NLG-1 with the *nlg-1* promoter (integrated transgene) did not impact aggregation behavior (**Fig. 3C**). Expressing sfGFP::NLG-1 in ADL (*srv-3* promoter) or ASH (*sra-6* promoter) individually or in AIA (*ins-1* promoter) did not rescue aggregation behavior (**Supplemental Fig. 2A**), but expression of sfGFP::NLG-1 in AIY neurons did increase the number of aggregating animals similar to levels with expression in all neurons (**Fig. 3C**). We also confirmed expression of all sfGFP::NLG-1 transgenes by analyzing expression by the sfGFP or GFP tag (**Supplemental Fig. 2B**). Together, these results imply that NLG-1 in neurons or in the AIY neurons is sufficient to partially modify aggregation behavior. The *nlg-1p* transgene surprisingly did not impact behavior, but may be overexpressed at a level that interferes with the behavior as these animals appear sluggish and somewhat slow growing." -- Page 8, lines 1-12

2. Figure 2D needs a label clearly indicating that the figure shows GFP::NRX-1 fluorescence.

We added the sfGFP label to make this figure clearer.

3. Throughout the study please clearly indicate when data are replotted. Please make clear in the methods section that appropriate corrections for multiple comparisons have been used.

We have tried to make this as clear as possible by adding specifics in the methods and figure legends describing where and what data is replotted. This is included in the methods text:
"Within figures and corresponding supplemental figures in which the same genotype(s) were used in multiple plots, data were replotted **(Fig. 2 & Supplemental Fig. 1, Fig. 3 & Supplemental Fig. 2, Fig. 4 & Supplemental Fig. 3)**. Data was not replotted between unrelated figures and supplemental figures." – Page 24, lines 9-12

And specific figure legends:

Example from Figure 2E "**E)** Graph showing number of aggregating animals in various genetic backgrounds. Data for *npr-1* and *npr-1;nrx-1* is plotted in both 2B and 2E." – Page 25, line 31

4. The authors' model predicts that mutation of glutamate receptors would have no effect in the absence of glutamatergic neurotransmission i.e. an eat-4 mutant. The authors should test this.

While our model may predict this, we do not state it since we did not directly test this possibility. As mentioned above, we identified 3 glutamate receptors that are involved and to truly test this hypothesis and model, we would need to combine the eat-4 mutation with all the receptor mutants individually, in combination, and all together. While we believe this could be interesting, this significant amount of work is beyond the scope of this manuscript and would not alter the main conclusions. We could include this speculation in the discussion section.

5. The authors refer to avr-15 as a glycine receptor homolog, which might be confusing as it is a glutamate-gated chloride channel. Also, it is not clear whether avr-15 is more similar to glycine or GABA receptors. If the authors wish to make the point that AVR-15 channels are possible GlyR homologs, it should be explained more clearly and better supported.

We recognize the confusion with the homolog identification as avr-15 is an invertebrate specific glutamate-gated chloride channel. We have labeled it as such clearly in the text (*avr-15 is an inhibitory glutamate-gated chloride channel)*. However, when searching for the closest human genes by amino acid sequence, the closest genes in humans are identified as GLRA2 and GABRB3 (which is shown in Supplementary Table 1). Further, in homolog searches through multiple databases (wormbase, flybase, ortholist 2) and in previous literature (Wong WR et al Hum Mol Genet. 2019), avr-15 is routinely compared to GLRA2 (39% identical) and GABRB3 (32% identical). While there is an early report that avr-15 does not function as a glycine or GABA receptor (10.1093/emboj/16.19.5867), it was performed *ex vivo* in *Xenopus* oocytes with only a single isoform and subunit expressed, and the data is not shown. Ligand specificity of these receptors can often depend on subunit composition and alternative splicing of isoforms, and therefore it is unclear how specific this channel is for glutamate vs other ligands as it has not been tested extensively. To further clarify, we have now added in the text "*avr-15* is an inhibitory glutamate-gated chloride channel[79] (whereas *GLRA2,GABRA3* are gated by glycine and GABA, respectively)" – Page 11, lines 4-6

6. The authors equate pHluorin recovery with glutamate release - this is not necessarily the case - this is an exocytosis assay and reflects the amount of membrane fusion.

We understand this concern and have added clarification on this point in the revised text. "which measures exocytosis and the amount of membrane fusion as a proxy measure for glutamate vesicle release" – Page 12, lines 1-2

7. Figure 5 is pixellated. Also, the cartoon depicting VGLUT::pHluorin makes VGLUT look like a channel, which might be confusing.

We have ensured this figure is now the same resolution as the other figures and have adjusted the cartoon to show a something more akin to a transporter rather than a channel.

8. Why was ADL ignored in pHluorin recovery experiments?

We have attempted to address this concern by making a strain that expresses the same phluorin construct as expressed in ASH, but in the ADL neurons. However, despite testing two ADL-specific promoters and multiple increasing concentrations of transgene injection, we were never able to visualize phluorin signal in the ADL neurons. While this was disappointing, we do not think this impacts the major findings or claims of the manuscript. A very recent report suggests that eat-4 is expressed very weakly in ADL, and perhaps it is under expression regulation at the protein level in this neuron (https://www.biorxiv.org/content/10.1101/2023.12.24.573258v2.full).

9. It isn't clear whether Figure 5D shows summary data from all experiments or representative data from one trial. Summary data of all the recovery slopes from all the trials should be shown in the main figures as scatter plots.

This is summary averaged data from all experiments, and we have added graphs with individual slopes for all animals in Supplemental Figure 4. We have edited the figure legend to make this clearly labelled.

10. The investigators measure effects of nrx-1 mutation on the number of CLA-1 puncta. What about size of puncta? Representative micrographs leave open the possibility that the size of puncta is affected.

We include analysis of the total area of puncta in addition to number of puncta for all comparisons but find no significant differences. This is included in Supplemental Figure 5 and in the text:
"We did not find differences in the total area of ADL or ASH pre-synaptic puncta in any genotypes compared to solitary controls or aggregating animals (**Supplemental Fig. 5).**" – Page 14, line 4-6

11. Why is CLA-1 signal affected by nrx-1 mutation but basal levels of EAT-4::pHluorin not? These are both presynaptic markers.

The analysis we include on eat-4::pHluorin intensity was not performed for cla-1, and the number and area of puncta analysis of cla-1 was not performed for eat-4::pHluorin as these experiments are looking at different aspects of synapses. However, it is not

uncommon for presynaptic markers to appear, localize, express, and be modified differentially – as shown recently by Kurshan et al for *rab-3* and *cla-1* in the DA9 neuron (https://pubmed.ncbi.nlm.nih.gov/30269993/), and McDonald et al for many endogenously tagged pre-synaptic components (https://pubmed.ncbi.nlm.nih.gov/33208945/).

12. What do asterisks in Figure 6E mean?

We have labelled asterisks in figure legend for 6E – "white asterisks indicate ASH cell body gfp expression and not synaptic puncta" – Page 27, lines 10-11

13. Figure 7 - these data seem to better fit in the earlier part of the narrative when the effect of nrx-1 mutation on aggregation is first reported. Also, it isn't clear that the effect of pkc-1(gf) will be modified by all the perturbations that modify npr-1(lf).

We understand this comment and have moved this experiment to Figure 1 and Supplemental Figure 1 and the corresponding results section.

14. The discussion point about an ancestral role for these genes in social behavior might be a bridge too far.

We agree that the comparisons we make are provocative on this idea in the discussion, we believe this is an important possibility to mention and will be of interest to many readers, but we are careful to not explicitly state that this is an 'ancestral role' for these genes.

Minor comments: We have edited all of these concerns as suggested
p. 10 line 5 reword - these data are consistent with prior observations
p. 11 line 23 reword 'trans-spliced GFP' should refer to coding sequences
p. 28 line 22 typo 'blech' should be 'bleach'

**Reviewer #1 (Remarks to the Author):**

The authors have performed most of the experiments I suggested. While the data they obtain from them do not neatly tie up the loose ends of the story, they are thought-provoking. They suggest considerable complexity in how this circuit functions.

We thank the reviewer for their helpful comments, and have added text in the discussion that comments on this considerable complexity from our findings.

**Reviewer #2 (Remarks to the Author):**

The authors already addressed my comments. I don't have further concerns.

We thank the reviewer for their helpful comments!

**Reviewer #3 (Remarks to the Author):**

The revised manuscript by Cowen and colleagues has been strengthened with additional data and by altering the structure of the narrative and edits to the text. My major comments have been addressed. I have notes on minor corrections that the authors can make.

(1) Page 4 lines 12-14 is awkwardly written and could confuse the reader.

We have edited the text to improve clarity:
"Despite *nrx-1* and *eat-4* (the vesicular glutamate transporter) both functioning in ASH and ADL sensory neurons to modulate aggregation behavior, they do so through distinct mechanisms. NRX-1 regulates an increase in the number of ASH pre-synaptic release sites in aggregating animals, while faster glutamate release from ASH neurons in aggregating animals occurs independently of NRX-1."

(2) Page 5 line 9 - the authors could convert the number of animals interacting into a fraction, i.e. 39 animals (out of 50) would instead be 78%.

We have now added the % of animals in the text:
"As expected, *npr-1(ad609)* mutants aggregate significantly more than solitary controls, with an average of 78% of *npr-1* animals aggregating compared to 4% of solitary control animals aggregating (*npr-1* average = 38.67, SEM= 1.675 vs. solitary control average =2.2, SEM =0.860, n = 50 animals)(**Fig. 1D&E**)."

(3) Page 6 line 7 - the authors refer to expression of pkc-1(gf) in neurons that provide peptidergic input to RMGs as 'outside the context of npr-1.' This is confusing. The experiment can (and probably should) be interpreted as indicating that gain-of-function in the NPR-1 signaling pathway causes similar effects regardless of whether RMGs or cells that regulate RMGs are targeted.

Apologies for the confusion. The PKC(gf) transgene has been used to activate neuronal activity and neurotransmitter/neuropeptide release of the RMG and upstream neurons, which induces aggregation behavior independently of npr-1 mutation. We have edited this sentence to clarify this point:
"Previous work found that activating sensory neurons and the RMG interneurons through expression of a constitutively active Protein Kinase C *(flp-21p::pkc-1(gf))* increases neurotransmission and induces aggregation behavior in solitary animals[21]. We used these animals to test whether *nrx-1* was needed for aggregation behavior induced independently of *npr-1* mutation. We found that, as previously reported, *flp-21p::pkc-1(gf)* induced social feeding, albeit at lower levels than *npr-1(ad609)* mutants[21] **(Fig. 1F ).**"

(4) Please check Results section for consistent use of the past tense.

We have edited the text to make the past tense consistent throughout.

(5) Please check figure legends for consistent use of 'data are' instead of 'data is.'

We have edited the figure legends to make this consistent throughout.

(6) The authors tend to over-hedge some statements, especially in the results. For example, on page 8 lines 15-18 the authors write that 'finding suggest' that genes 'likely function in parallel.' It suffices to write that the findings suggest parallel action. Please check the text for other instances of this over-hedging.

We have identified a number of relevant sections and edited to remove over-hedging:
Page 7, lines 1-2: "Collectively, these data suggest that NRX-1(α) functions in two pairs of sensory neurons for aggregation behavior."

Page 8 lines 8-9 "Together, these results imply that NLG-1 in all neurons or in the AIY interneurons to partially modify aggregation behavior."

page 8 lines 15-18 "These findings suggest that both *nrx-1* and *nlg-1* are critical for aggregation behavior, but function in parallel, non-epistatic, molecular pathways."

page 9 lines 15-16 "This result indicates that glutamate and *nrx-1* function in parallel, non-epistatic, pathways to affect aggregation behavior."

page 9 line 21 "Therefore, we conclude that (1) multiple molecular signaling components contribute to aggregation behavior, (2) that *nrx-1* functions in genetically distinct or parallel pathways to *nlg-1* and *eat-4*, (3) while *nlg-1* and *eat-4* function in partially overlapping pathways."

Page 14 lines 6-9 “The *nrx-1* dependent changes in ASH puncta suggest that *nrx-1* mutations prevent the conversion of solitary to more social behavior through a reduction in glutamate input to other neurons (i.e. ADL, RMG), which lowers circuit activity and aggregation behavior (**Fig. 1F**).”

(7) Page 13 line 3 - the description of CLA-1 imaging is confusing. First, the authors refer to the method used as 'enhanced resolution confocal microscopy.' What does this mean? Second, the authors write that they image a 'chemical GFP-tagged pre-synaptic marker.' What does this mean? My understanding was that these experiments involved conventional imaging of GFP-tagged CLA-1. Did I miss something?

We apologize for the confusion, we have edited the text to clarify the meaning, and removed chemical, which was out of place referring to chemical synapses specifically: “To investigate whether *nrx-1* alters aggregation behavior through a role in synaptic structure or architecture, we analyzed pre-synaptic morphology of the ADL and ASH neurons using enhanced resolution confocal microscopy (Leica Lightning Deconvolution analysis). Specifically, we used a GFP-tagged pre-synaptic marker clarinet CLA-1 (a bassoon ortholog) and quantified CLA-1::GFP puncta in the neurites of ADL or ASH sensory neurons using the *srv-3* and *sra-6* promoters via an unbiased particle analysis (see methods for details, **Fig. 6A**) [84].

(8) I encourage the authors to use more of the Discussion section to synthesize their observations into models and speculate. As written, the Discussion section devotes a little too much bandwidth to summarizing the study.

We appreciate this comment and have worked to edit and improve the discussion. We removed some text summarizing the study and our findings, and added sentences synthesizing observations into models and speculating on what future work could develop these findings further. This relatively significant editing is marked via track changes in the manuscript document.