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Feeding state regulates pheromone-mediated avoidance behavior via the insulin signaling pathway in *C. elegans*

Leesun Ryu, YongJin Cheon, Yang Hoon Huh, Seondong Pyo, Satya Chinta, Hongsoo Choi, Rebecca A. Butcher, and Kyuhyung Kim

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Transaction Report:

(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. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 23 November 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. We have now received three referee reports on your manuscript, which I am copying below for your information.

As you can see from the comments, all three referees express interest in the work and the topic. However, the referees #1 and #2 also raise several substantial concerns that need to be addressed in order to consider publication here. Given the referees' positive recommendations, I would like to invite you to submit a revised version of your manuscript in which you address the comments of all three referees.

I should add that it is The EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the main concerns raised at this stage.

We generally allow three months as standard revision time. Please contact us in advance if you would need an additional extension. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work to discuss how to proceed.

Please feel free to contact me if have any further questions regarding the revision. Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Referee #1:

This paper investigates the effect of feeding state on aversive responses to pheromone in C. elegans. The authors find that starved animals show increased avoidance of the pheromone ascr#3 compared

to well-fed worms, and this effect is dependent on the insulin receptor daf-2. daf-2 mutants also show decreased ascr#3 avoidance generally, and this effect is due to reduced synaptic transmission from the primary sensory neuron ADL (where DAF-2 is required) and first-order interneurons. They further show that an intesinal insulin-like peptide, INS-18, also modulates ascr#3 avoidance; genetic epistasis experiments imply that it functions upstream of DAF-2 in an inhibitory manner. Since INS-18 release is shown to be reduced in starved worms, this suggests a hormonal signaling mechanism for feeding-state-dependent modulation of behavior.

Overall, I think this is a very interesting story, with novel implications about the role of endocrine-neuronal interactions in this important model organism. There are a couple of places where I think the authors could fill gaps in the story that would make it more complete and logically compelling.

1. The authors show that daf-2 mutants fail to increase their avoidance of pheromone upon starvation, but the avoidance in well-fed animals is also very low. Thus, it is difficult to say that DAF-2 signaling is involved in food modulation as opposed to being generally required for ADL neurotransmission. One experiment that might get at this issue would be to look at daf-16 mutants in both starved and unstarved condiditions. If daf-16 mutants show comparably high avoidance under both conditions, this would suggest that the ability to turn the pathway up or down is necessary for modulation.

2. Another question, which the authors avoid making a strong statement on, is whether DAF-2 is actually the receptor for INS-18. Indeed the authors' data doesn't really address whether INS-18 acts on ADL, the cell in which DAF-2 functions for this behavior. If the authors were able to do calcium imaging experiments in ADL and AVA in ins-18 mutants, as they did with daf-2 and wild-type animals, this might shed some light on this.

3. Finally, a minor suggestion--it might be interesting to do the ascr#3 avoidance assays ON food as well as off food, to distinguish affects of starvation from effects of food availability.

Referee #2:

In this manuscript, Leesun et.al described a nice study on how diet availability modulates internal metabolic pathways to shape sensory perception in C. elegans. They found that prolonged starvation could decrease the release of INS-18 from C. elegans intestine and relieve the antagonistic effect of INS-18 on DAF-2 receptor in ADL neurons, which would increase ADL synaptic output to AVA command interneurons and thus potentiate ascr#3-induced avoidance behavior. They suggested that this mechanism would help the worm in overcrowding and starvation conditions to explore a new environment. Overall, the authors presented an interesting story; however, a number of key questions need to be addressed to make their conclusions solid.

1) The fluorescence quantification methods they used in this study are not acceptable. Grading the fluorescence brightness by naked eyes is not objective.

2) In addition, extra-chromosomal array transgenic worms are used to do the fluorescence quantification analysis in this study, which makes their results not reliable, because the expression level of extra-chromosomal array is mosaic. Integrated transgenic lines are more desirable for this type of quantifications.

3) Previous studies have shown that starvation decreases DAF-2 activity and promotes DAF-16 nuclear translocation in ADL neuron in a cell-autonomous manner, which are kind of opposite to the results shown in this study. The authors shall perform more experiments to resolve this discrepancy, instead of simply claiming "a multitude of complexity in the function of and/or gene expression in the ADL sensory neurons".

4) As the authors discussed, besides INS-18, INS-1 is also expressed in intestinal cells. Is INS-1 release from the intestine modulated by external food availability?

5) As several studies have shown that Psre-1::GFP not only labels ADL but also ASJ neurons, the author should generate and test other rescuing transgenic lines with a different promoter to confirm

their results.

6) The authors only used fraction of reversing to quantify ascr#3 avoidance behavior. It would be more informative to include the quantification of reversal delay time and reversal distance.

7) In fig2, the authors only presented the calcium response of WT and daf-2 mutants. How about well-fed and starved WT and daf-2 mutants?

8) The authors claimed that DAF-2 regulates synaptic transmissions in ADL. However, they only presented the intensity decrease of SNB-1::YFP or RAB-3::YFP. These data are not enough to support the conclusion of synaptic transmission decrease in ADL. To make such a claim, the authors shall provide functional data by directly monitoring presynaptic release using electrophysiological recording or PHluorin imaging.

9) To examine the presynaptic function of ADL, the authors measured fluorescence intensity of SNB-1::YFP in areas near the nerve ring where ADL and AVA connect each other. However, they did not indicate the particular area where ADL and AVA connect with each other. Actually, it is impossible to do that using ADL::SNB-1::YFP (ADL makes a lot more synapses with AIB and AVD). One way to identify the specific connections between ADL and AVA is to use GFP Reconstitution Across Synaptic Partners (GRASP).

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11) In fig 5, the authors used a pan-neural unc-14 promoter to perform ins-18 rescuing experiments. The negative results could be explained by network imbalance because ins-18 is ectopically expressed in many other neurons. To rule out the possibility of ins-18 functioning in neurons, the authors may want to use its own promoter.

12) In fig 5D, the two sample pictures look different. Why?

13) In fig 6, the authors temporarily knocked down ins-18 function for 6 h and 24 h using RNA interference (RNAi). It would be nice if he authors can determine whether and how these two RNAi protocols work differently in terms of efficiency/potency.

Referee #3:

Feeding state influences pheromone-mediated avoidance behavior via the insulin signaling pathway in C. elegans

In this paper, Ryu et al. investigated the mechanisms whereby internal states, particularly feeding states, regulated a sensory response in C. elegans. They showed that food-deprivation increased the avoidance of a pheromone ascr#3 in a daf-2-dependnet manner. They also showed that the daf-2 signaling pathway regulated the avoidance of ascr#3 in both well-fed and starved animals. They found that daf-2 modulated the avoidance by acting in the sensory neurons ADL, which they previously showed to respond to ascr#3. The further showed that daf-2 acted through the canonical IGF pathway in ADL to downregulate the presynaptic protein snb-1 and that the daf-2 mutation decreased the expression of snb-1 in ADL and reduced the activity of the command interneuron AVA, a downstream interneuron that promoted reversals. They identified ins-18 as the ligand of the daf-2 signaling pathway, which was secreted from the intestine to regulate avoidance of ascr#3. Starvation decreased the secretion of ins-18, which modulated ADL response to ascr#3 via daf-2.

The study investigated an important neurobiology question. The experiments were well designed and executed. The results are convincing and complete. The findings are mechanistic and important.

I only have a few remarks, which will help to improve the presentation of the results.
1. Different concentrations of ascr#3 were used in several experiments. The rationale needs to be better explained.
2. In different experiments, the "fraction reversing" appears to vary greatly. It will be helpful if the authors discuss the potential conditions that can potentially contribute to the variations.
3. "DAF-2 signaling influences Ca2+ responses, not in the ADL sensory neurons, but in the AVA interneurons upon ascr#3 exposure." "influences" appears to be a weak expression in this context, the authors may want to consider to use "mediates" or "regulates".
4. Figure 2c, should the labeling "F/F" be "deltaF/F"?
5. For fig 2, the authors need to clarify how deltaF/F was calculated. In addition, how was the average peak "deltaF/F" quantified?
6. Some images in fig 3a are difficult to see.
7. Is the snb-1cNDA::yfp functional? If the authors have the data to demonstrate this, it should be included. Otherwise, the authors should discuss potential limitations of the approach.
8. The authors used bar graphs in some figures, but box plots in others. What is the rationale for using different formats for the data presentation?
9. Similarly, is the ins-18 cDNA::gfp functional? If the authors do not have additional data to address the question, the potential limitations should be discussed.

1st Revision - authors' response 7 March 2018

Summary

We have substantially revised the manuscript, and performed additional experiments to address the reviewers' comments. New data are now presented in Figures 4C, EV1A, EV1D, EV1E, EV2A, EV2B, EV2C, EV2F, and EV4B. We include additional authors (Y. Cheon and YH Huh) who performed additional experiments.

We are grateful to the reviewers for their valuable comments. Responses to specific points are provided below.

Referee #1:

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***1.** The authors show that daf-2 mutants fail to increase their avoidance of pheromone upon starvation, but the avoidance in well-fed animals is also very low. Thus, it is difficult to say that DAF-2 signaling is involved in food modulation as opposed to being generally required for ADL neurotransmission. One experiment that might get at this issue would be to look at daf-16 mutants in both starved and unstarved conditions. If daf-16 mutants show comparably high avoidance under both conditions, this would suggest that the ability to turn the pathway up or down is necessary for modulation.*

We thank the reviewer for these excellent suggestions. As suggested by the reviewer, we performed ascr#3 avoidance assay in *daf-16* mutants under well fed as well as starved for 3 h, 6 h, or 24 h

conditions. We found that increased ascr#3 avoidance was maintained in starved conditions (New Figure 4C), suggesting that DAF-2/DAF-16 signaling pathway mediates feeding state-dependent modulation of ascr#3 avoidance.

2. *Another question, which the authors avoid making a strong statement on, is whether DAF-2 is actually the receptor for INS-18. Indeed, the authors' data doesn't really address whether INS-18 acts on ADL, the cell in which DAF-2 functions for this behavior. If the authors were able to do calcium imaging experiments in ADL and AVA in ins-18 mutants, as they did with daf-2 and wild-type animals, this might shed some light on this.*

We are pleased that the reviewer raised this concern. In *C. elegans*, INS-18 appears to act as a DAF-2 antagonist for dauer formation and longevity (Matsunaga, Gengyo-Ando et al., 2012; Fernandes de Abreu, Caballero et al., 2014). Our results indicate that INS-18 may act as a DAF-2 antagonist for feeding state-dependent ascr#3 avoidance behaviors. Taken together, INS-18 could antagonize most, if not all, DAF-2 functions. As pointed out by the reviewer, the next obvious question is whether INS-18 is indeed cognate ligands for DAF-2. Functional expression of *C. elegans* proteins, especially transmembrane receptor proteins, in heterologous expression systems is notoriously difficult, which hampers our examination for direct interaction between INS-18 and DAF-2. Therefore, we would like to, if possible, further investigate these ligand receptor interactions in future studies.
However, we specifically tried to address this issue in the manuscript. In Figure 6E, we showed that *daf-2* mutation suppressed the increase of ascr#3 avoidance in *ins-18* RNAi-treated worms, and the expression of *daf-2* cDNA in ADL restored the enhanced ascr#3 avoidance in *ins-18* RNAi-treated *daf-2* mutants, suggesting that INS-18 regulates the DAF-2 signaling pathway in the ADL neurons. As the reviewer suggested, we tested this issue further by performing *in vivo* calcium imaging experiments in the ADL and AVA neurons of *ins-18* mutants (Figure 1 here). Similar to *daf-2* mutants, Ca^{2+} transients of ADL upon 500 nM ascr#3 exposure were not altered in *ins-18* mutants (Figure 1A here). However, Ca^{2+} transients of AVA upon 500 nM ascr#3 exposure were actually *not increased* in *ins-18* mutants (Figure 1B here), which was unexpected. Then, we further found that both 100 nM and 500 nM ascr#3 exposure exhibited similar Ca^{2+} responses of AVA (Figure 1D here), indicating that AVA Ca^{2+} responses to ascr#3 exposure may be saturated. Although we cannot completely rule out that results of AVA Ca^{2+} responses in *ins-18* mutants are indeed correct, we believe that AVA Ca^{2+} responses to ascr#3 exposure may be not good readouts for *increased* synaptic activities from ADL to AVA. Similarly, we could not detect *increased* Ca^{2+} responses of AVA upon ascr#3 exposure in starved conditions (Figure 1C here). We chose to not include these data in the manuscript since we wished to keep the work focused on not a caveat of Ca^{2+} imaging in a specific neuron but roles of INS-18.

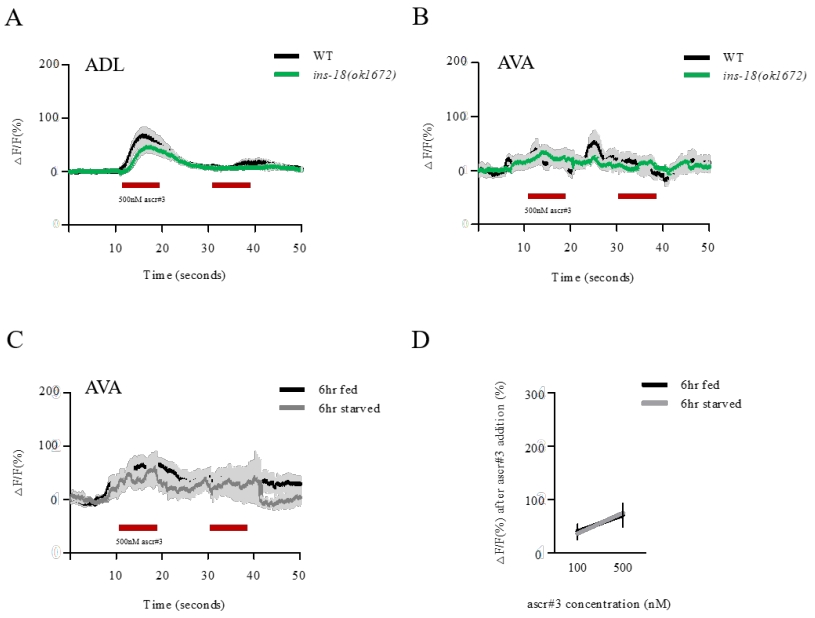

Figure 1 Ca^{2+} transients of the ADL and AVA neurons in *ins-18* mutants and feeding conditions

A-B. Ca^{2+} transients of ADL (A) and AVA (B) upon 500 nM ascr#3 exposure in wild-type and *ins-18* mutant animals. n = 7–12.

C-D. Ca^{2+} transients to 500 nM ascr#3 of AVA (C) and the dose-response curve of the average peak percentage changes in fluorescence Ca^{2+} peaks upon 100 nM and 500 nM ascr#3 exposure (D). n = 7–11. All error bars rep

3. *Finally, a minor suggestion--it might be interesting to do the ascr#3 avoidance assays ON food as well as off food, to distinguish effects of starvation from effects of food availability.*

As suggested by the reviewer, we tested ascr#3 avoidance behavior ON food. Previously, we and others showed that ascr#3 avoidance behaviors were enhanced in the presence of food (Jang, Kim et al., 2012). Here we recapitulate previous observation (New Figure EV1D). Furthermore, increased ascr#3 avoidance behaviors ON food were also abolished in *daf-2* mutants (New Figure EV1D), suggesting that DAF-2 signaling modulates feeding state-dependent alteration of ascr#3 avoidance.

Referee #2:

In this manuscript, Leesun et.al described a nice study on how diet availability modulates internal metabolic pathways to shape sensory perception in C. elegans. They found that prolonged starvation could decrease the release of INS-18 from C. elegans intestine and relieve the antagonistic effect of INS-18 on DAF-2 receptor in ADL neurons, which would increase ADL synaptic output to AVA command interneurons and thus potentiate ascr#3-induced avoidance behavior. They suggested that this mechanism would help the worm in overcrowding and starvation conditions to explore a new environment. Overall, the authors presented an interesting story; however, a number of key questions need to be addressed to make their conclusions solid.

1. *The fluorescence quantification methods they used in this study are not acceptable. Grading the fluorescence brightness by naked eyes is not objective.*

Taken into account the reviewer's point, we re-measured fluorescence intensity using ImageJ software (https://imagej.nih.gov/ij/index.html). We analyzed fluorescence intensity of SNB-1::YFP and mCherry::RAB-3 in the ADL processes of wild-type and *daf-2* mutant animals using ImageJ and found that these new data (New Figure EV2B, EV2C) are very comparable to the original data (Fig 3B, 3C). Thus, we are now comfortable with the fluorescence quantification data presented in current Figures.

2. *In addition, extra-chromosomal array transgenic worms are used to do the fluorescence quantification analysis in this study, which makes their results not reliable, because the expression level of extra-chromosomal array is mosaic. Integrated transgenic lines are more desirable for this type of quantifications.*

As the reviewer is aware, transgenic animals expressing extrachromosomal arrays often exhibit partial and variable transmission rates as well as mosaic transgene expression (Mello, Kramer et al., 1991). One way to overcome this issue is to integrate extrachromosomal arrays into the genome, expecting more stable and consistent expression of transgenes. However, the integrated lines have own limitations. During integration, animals need to be exposed to gamma-ray or ultraviolet irradiation which creates a number of unlinked mutations in their genome anywhere. Furthermore, transgenes could be inserted on a genome near or on-site to the gene of interest that lead to undesired phenotypes. Thus, in some cases analysis of transgenic lines expressing extrachromosomal arrays is more beneficial compared to that of integrated lines.

As suggested by the reviewer, we integrated extrachromosomal arrays expressing *sre-1*p*::snb-1* cDNA*::yfp* transgene by UV irradiation and examined expression of SNB-1::YFP in the ADL processes of wild-type and *daf-2* mutant animals (New Fig EV2A). We were able to recapitulate the original extrachromosomal array data (Fig 3B) with an integrated line. Thus, again we are now comfortable with the extrachromosomal array data presented in current Figures.

3. *Previous studies have shown that starvation decreases DAF-2 activity and promotes DAF-16 nuclear translocation in ADL neuron in a cell-autonomous manner, which are kind of opposite to*

the results shown in this study. The authors shall perform more experiments to resolve this discrepancy, instead of simply claiming "a multitude of complexity in the function of and/or gene expression in the ADL sensory neurons".

We are pleased that the reviewer raised this point. To address this point, we now have monitored DAF-16 localization in ADL under different feeding conditions using transgenic animals expressing *sre-1*p*::gfp::daf-16*a cDNA. As reported in Gruner et al., 2016, we observed that GFP::DAF-16 was highly accumulated in the nucleus of ADL in 12 h starvation (Figure 2 here). However, we also noticed that DAF-16 was localized near nuclear membrane of ADL in 1 to 6 h starvation (Figure 2 here), and this nuclear membrane localization was disappeared in 12 h starvation. We think that nuclear membrane localization of DAF-16 may have roles in feeding state-dependent ascr#3 avoidance behavior. Recently, roles of nuclear membrane location of transcription factors have been reported (Heessen & Fornerod, 2007). However, roles of DAF-16 on nuclear membrane of ADL are not clear and need to be investigated further. Thus, we would like to study, if possible, this issue further in future studies.

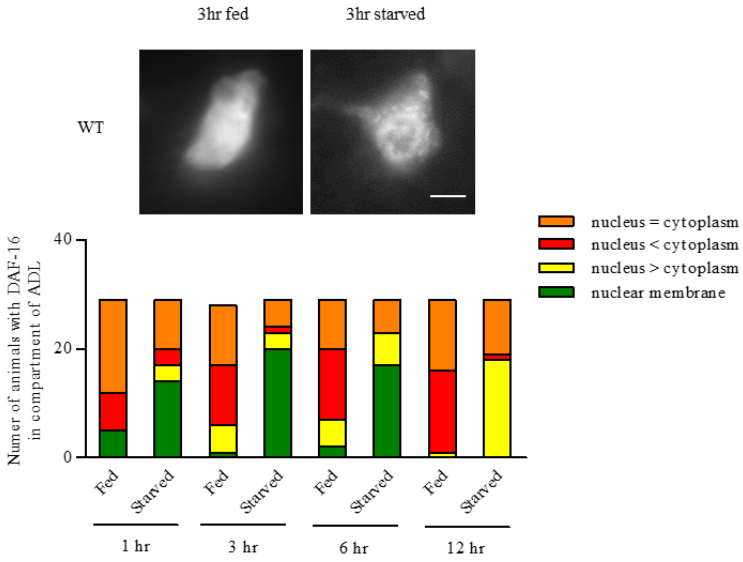

Figure 2. Representative images of transgenic animals expressing *sre-1*p*::gfp::daf-16* cDNA at 3hr fed and starved conditions (upper) and the number of animals with DAF-16 in component of ADL under feeding conditions (lower). n = 29. White scare bar is 0.5nm.

4. *As the authors discussed, besides INS-18, INS-1 is also expressed in intestinal cells. Is INS-1 release from the intestine modulated by external food availability?*

As suggested by the reviewer, we now generated transgenic animals expressing *acd-5*p*::ins-1* cDNA*::gfp* transgene and examined intestinal INS-1 release under fed and starved conditions. Opposed to decreased INS-18 secretion, INS-1 release was actually increased from intestine in 6 hr starvation (Figure 3 here), suggesting that INS-1 release from the intestine is also modulated by feeding conditions. However, as shown in Fig 5B, *ins-1* appears to function in parallel to or downstream of *daf-2/daf-16* to regulate ascr#3 avoidance. Therefore, we chose to not include these data in the manuscript since we wished to keep the work focused on roles of not INS-1 but INS-18 as upstream molecules of DAF-2 signaling and would prefer to investigate roles of INS-1 in feeding state-dependent ascr#3 avoidance in future studies.

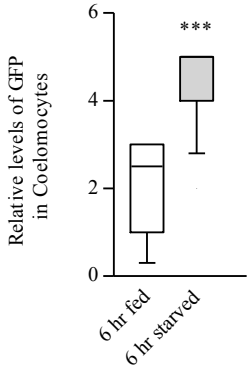

Figure 3. Relative fluorescence intensity of the accu mulated GFP in transgenic animals expressing *acd-5* p*::ins-1* cDNA*::gfp* in coelomocytes. n = 12–13. ** * p < 0.001 (unpaired student's t-test).

5. *As several studies have shown that Psre-1::GFP not only labels ADL but also ASJ neurons, the author should generate and test other rescuing transgenic lines with a different promoter to confirm their results.*

Previously, the promoter of the *sre-1* gene has been shown to drive transgene expression specifically in the ADL as well as ASJ neurons of the head of worms (Troemel, Chou et al., 1995). However, after we empirically noticed that the *sre-1* promoter is predominantly expressed in ADL than ASJ, we and others have been using the *sre-1* promoter as an ADL specific promoter for expressing transgenes in the ADL neurons (Jang et al., 2012; Gruner, Nelson et al., 2014; Gruner, Grubbs et al., 2016; Hong, Ryu et al., 2017). To address this issue raised by the reviewer, we carefully examined the expression pattern of transgenic animals expressing *gfp* transgene under the control of the *sre-1* promoter. We found that as expected, GFP is predominantly expressed in the ADL neurons (New Fig EV1E), supporting that *sre-1* promoter can be used as an ADL specific promoter.

6. *The authors only used fraction of reversing to quantify ascr#3 avoidance behavior. It would be more informative to include the quantification of reversal delay time and reversal distance.*

In the avoidance assay to measure responses upon exposure of chemical repellants which was originally developed by (Hilliard, Bargmann et al., 2002), we and others have quantitated avoidance behaviors by separating three categories: short reversal, long reversal, and omega turn (Ibsen, Tong et al., 2015; Hong et al., 2017). Specifically, short or long reversals were defined as reversals with fewer than two head bends or more than two head bends, respectively. In this study, we counted long reversals as repulsion when occurred within 4 seconds after ascr#3 exposure because we noticed that short reversals and omega turn were not affected by feeding conditions (New Fig EV1A). As suggested by the reviewer, we now showed the quantification data of short reversals and omega turn in New Fig EV1A. We also modified details of the avoidance assay in the Method.

7. *In fig2, the authors only presented the calcium response of WT and daf-2 mutants. How about well-fed and starved WT and daf-2 mutants?*

For AVA Ca^{2+} imaging experiment in wild-type animals, please see responses to reviewer 1's 2nd comments.
Previously, Gruner et al., 2014 showed that Ca^{2+} transients of ADL upon ascr#3 exposure were not affected by 6 hr starvation. We now modified main text and added this reference.
Since *daf-2* mutants exhibit almost NO Ca^{2+} transients of AVA upon ascr#3 exposure in fed condition, we did not expect further decrease of Ca^{2+} transients of AVA of *daf-2* mutants upon ascr#3 exposure in starved conditions.

8. *The authors claimed that DAF-2 regulates synaptic transmissions in ADL. However, they only presented the intensity decrease of SNB-1::YFP or RAB-3::YFP. These data are not enough to support the conclusion of synaptic transmission decrease in ADL. To make such a claim, the authors shall provide functional data by directly monitoring presynaptic release using electrophysiological recording or PHluorin imaging.*

The reviewer raised the valid issue. As shown in Fig 3F, we actually manipulated synaptic outputs of the ADL neurons by expressing tetanus toxin light chain (TeTx) or gain-of-function mutation of *pkc-1* protein kinase C gene, and showed that synaptic transmission is altered in ADL of *daf-2* mutants. However, to address this issue further, as suggested by the reviewer, we now performed VGLUT-pHluorin imaging of which technique is recently developed and validated in *C. elegans* (Ventimiglia & Bargmann, 2017). We generated transgenic animals expressing *sre-1*p*::eat-4*(VGLUT) cDNA::pHluorin and tested whether we can detect fluorescence changes upon ascr#3 exposure. We were able to detect *little* change of fluorescence upon over 2000 nM ascr#3 exposure (Figure 4 here) which is not physiological concentrations used. Unfortunately, we did not see any responses upon 100, 200, 500 nM ascr#3 exposure probably due to low sensitivity of this system, which makes us to stop further examination. In addition, the electrophysiology recording in *C. elegans* neurons has own limitations (Goodman, Lindsay et al., 2012). Thus, we would like to investigate, if possible, this issue further in future studies.

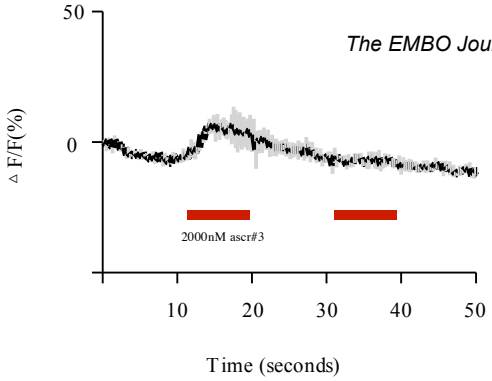

Figure 4. VGLUT-pHluorin imaging of ADL upon 2000 nM ascr#3 exposure. n = 3 (Among 10 animals test ed, only 3 worms showed responses)

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The reviewer again raised the valid issue. To address this issue, as suggested by the reviewer, we now used a genetically encoded fluorescent trans-synaptic marker called NLG-1 GRASP (Neuroligin-1 GFP reconstitution across synaptic partners) which labels synapses between individual neurons of interest (Park, Knezevich et al., 2011). We expressed NLG-1 GRASP markers in ADL (*sre-1p::nlg-1::spGFP1-10*) as well as AVA (*nmr-1p::nlg-1::spGFP11*). Although we generated many transgenic lines, we did not see any GRASP signals in the nerve ring where ADL-AVA synaptic connections exist. We now noticed that to obtain reliable GRASP signals needs many trials and errors (Oren-Suissa, Bayer et al., 2016). Then we further addressed this issue by performing electron microscopy reconstruction. Although it is time-consuming and labor-intensive, electron microscopy is a powerful tool to identify synaptic structures. We now tried conventional transmission electron microscopy (TEM) as well as high voltage electron microscopy (HVEM) to see the ADL-AVA connections. However, we ended up to acquire very low resolution images in which we could not identify the ADL-AVA connections. This may be due to lack of previous experience for sampling of *C. elegans*. Thus, again we would like to investigate, if possible, this issue further in future studies.

10. *In fig 3, the authors found that the intensity of both SNB-1::YFP and RAB-3::YFP is decreased in daf-2 mutants. It will be good if the authors could add a negative control such as calcium channel::YFP.*

The reviewer raised the valid point. To address this issue, we now examined the fluorescence level of OCR-2::mCherry in *daf-2* mutants. *ocr-2* encodes a TRPV channel that acts in the ADL neurons to mediate ascr#3 avoidance (Jang et al., 2012). We generated transgenic animals expressing *sre-1p::ocr-2* genomic*::mcherry* transgene and found that OCR-2 level was not altered in *daf-2* mutants (New Fig EV2F), indicating that OCR-2::mCherry can be used as a negative control for these assays.

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As the reviewer pointed out, the negative results in rescue experiments generated by using broadly expressed promoters could be explained by several reasons including network imbalance. Expression pattern of *ins-18* gene was previously determined; *ins-18* is expressed in many head and tail neurons and other tissues including intestinal cells (Pierce, Costa et al., 2001; Matsunaga et al., 2012; Ritter, Shen et al., 2013; Hung, Wang et al., 2014), which prevented us from using the *ins-18* promoter for neuronal expression. Instead, to address this problem, we used another pan-neural *rgef-1* promoter and found that the ascr#3 avoidance phenotype of *ins-18* mutants was again not rescued

(New Figure EV4B), supporting that *ins-18* does not act in the neurons to mediate ascr#3 avoidance behaviors.

12. *In fig 5D, the two sample pictures look different. Why?*

Right and left pictures of fig 5D represent expression levels of SNB-1::YFP in wild-type and *ins-18* mutant animals, respectively. These pictures were taken at the same day under the same conditions. We are not sure of the nature of those difference but it is probably due to backgrounds from very low levels of SNB-1::YFP expression.

13. *In fig 6, the authors temporarily knocked down ins-18 function for 6 h and 24 h using RNA interference (RNAi). It would be nice if he authors can determine whether and how these two RNAi protocols work differently in terms of efficiency/potency.*

We intended to recapitulate starvation effects on ascr#3 avoidance and intestinal INS-18 release by temporarily knocking down *ins-18* function by RNAi. Although starvation for 6 h was sufficient for increase of ascr#3 avoidance and decrease of intestinal INS-18 release, *ins-18* RNAi for 6 h did not alter intestinal INS-18 release. We believed that this is probably due to insufficient time for RNAi for 6 h to mimic starvation for 6 h. Therefore, we decided to extend RNAi treatment time for 24 hr (Kamath, Fraser et al., 2003) and then found comparable results. We have now tried to explain this in text.

Referee #3:

In this paper, Ryu et al. investigated the mechanisms whereby internal states, particularly feeding states, regulated a sensory response in C. elegans. They showed that food-deprivation increased the avoidance of a pheromone ascr#3 in a daf-2-dependant manner. They also showed that the daf-2 signaling pathway regulated the avoidance of ascr#3 in both well-fed and starved animals. They found that daf-2 modulated the avoidance by acting in the sensory neurons ADL, which they previously showed to respond to ascr#3. The further showed that daf-2 acted through the canonical IGF pathway in ADL to downregulate the presynaptic protein snb-1 and that the daf-2 mutation decreased the expression of snb-1 in ADL and reduced the activity of the command interneuron AVA, a downstream interneuron that promoted reversals. They identified ins-18 as the ligand of the daf-2 signaling pathway, which was secreted from the intestine to regulate avoidance of ascr#3. Starvation decreased the secretion of ins-18, which modulated ADL response to ascr#3 via daf-2. The study investigated an important neurobiology question. The experiments were well designed and executed. The results are convincing and complete. The findings are mechanistic and important. I only have a few remarks, which will help to improve the presentation of the results.

1. *Different concentrations of ascr#3 were used in several experiments. The rationale needs to be better explained.*

As the reviewer may know, behaviors have been difficult to study because behavioral outputs could be affected by endogenous noise as well as ever-changing environmental conditions (Stern, Kirst et al., 2017). ascr#3 avoidance behaviors are also modulated by food availability, previous experience, and feeding conditions (Jang et al., 2012; Hong et al., 2017; in this study). We also noticed that the quality and quantity of ascr#3 chemicals make data of avoidance behavior to be variable. To overcome these problems, we empirically chose one concentration of ascr#3 for generation of more consistent behavioral data; 100 nM ascr#3 for the assay under different feeding conditions and 500 nM ascr#3 for the assays of mutants in fed status. We now explained this in the Method.

2. *In different experiments, the "fraction reversing" appears to vary greatly. It will be helpful if the authors discuss the potential conditions that can potentially contribute to the variations.*

Please see responses to 1st comments above. We now explained this in the Method.

3. *"DAF-2 signaling influences Ca2+ responses, not in the ADL sensory neurons, but in the AVA interneurons upon ascr#3 exposure." "influences" appears to be a weak expression in this context, the authors may want to consider to use "mediates" or "regulates".*

Changed (Thank you)

4. *Figure 2c, should the labeling "F/F" be "deltaF/F"?*

Changed (Thank you)

5. *For fig 2, the authors need to clarify how deltaF/F was calculated. In addition, how was the average peak "deltaF/F" quantified?*

We followed the previously described protocol for quantification of fluorescence changes (Jang et al., 2012). The cell body area of an ADL neuron was selected as the region of interest and a similar sized area near the cell body was selected as the background. Average fluorescence intensities in the first 5 seconds of imaging were used for normalization to calculate ΔF/F. The peak of "ΔF/F" represented maximum ΔF/F during the first ascr#3 exposure and we averaged these data to obtain the average peak of ΔF/F. We now explained this better in the Method.

6. *Some images in fig 3a are difficult to see.*

We now increased brightness of both pictures in Fig 3A at the same extent.

7. *Is the snb-1cDNA::yfp functional? If the authors have the data to demonstrate this, it should be included. Otherwise, the authors should discuss potential limitations of the approach.*

Yes, it was previously shown that the same *snb-1* cDNA*::yfp* which we used in this study is functional (Nonet, Holgado et al., 1999; Shen & Bargmann, 2003; Noma & Jin, 2015). We now modified the text accordingly.

8. The authors used bar graphs in some figures, but box plots in others. What is the rationale for using different formats for the data presentation?

We presented ascr#3 avoidance (fraction reversing) in bar graph and fluorescence quantification in box plots to present the data more clearly.

9. Similarly, is the ins-18 cDNA::gfp functional? If the authors do not have additional data to address the question, the potential limitations should be discussed.

Yes, it was also previously shown that the same *ins-18* cDNA*::gfp* which we used in this study is functional (Hung et al., 2014). We now modified the text accordingly.

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2nd Editorial Decision 6 April 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by three of the original referees whose comments are shown below.
As you will see they all find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address.

1. Please address the 3rd comment of the referee #3 by changing the title accordingly. I see that the running title was changed already.
2. Please mention the functionality of the constructs used in the text, as pointed out by referee #3 in points 7 and 9.

Referee #1:

I think the authors have done a good job addressing my suggestions as well as those of the other reviewers. I am supportive of publication.

Referee #2:

The authors have done a good job in revising the manuscript. Though they are unable to obtain positive data or perform experiments to address some of my comments due to technical challenges, overall the study is quite comprehensive and mechanistic. I am therefore happy to support its publication in EMBOJ. Congratulations on a nice piece of work!

Referee #3:

To revise the manuscript, the authors performed a substantial amount of experiments to address concerns raised by the reviewers. A couple of the experiments depend on methods that are well known to be technically challenging. The new results generated by the additional experiments are compelling and further strengthen the original findings. The authors also improved the clarity of the presentation. In my views, the manuscript is now ready to be published on the EMBOJ.

2nd Revision - authors' response 10 May 2018

1. Please address the 3rd comment of the referee #3 by changing the title accordingly. I see that the running title was changed already.

Changed

2. Please mention the functionality of the constructs used in the text, as pointed out by referee #3 in points 7 and 9.

I inserted ‘functional YFP-tagged SNB-1/synaptobrevin’ and ‘functional acd-5p::ins-18 cDNA::gfp in the text’

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Kyuhyung Kim
Journal Submitted to: The EMBO journal
Manuscript Number: EMBOJ-2017-98402

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.

A- Figures

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?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - 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)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	we followed up C. elegans research standard for choosing the sample size
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For behavioral analysis, we used more than 30 worms and for imaging experiments, we used more than 5 worms.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We included all animals tested.
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.	No
For animal studies, include a statement about randomization even if no randomization was used.	We randomely chose animals from populations.
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.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	For behavioral assay, we have done blinding.
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.	Yes, see the methods
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
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http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

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http://www.ncbi.nlm.nih.gov/gap

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http://www.selectagents.gov/

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).	NA
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

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.	C. elegans hermaphrodites were used in this study. They are grown on NGM agar plates fed with OP50.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
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.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

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	All DNA seuqences are already avaiable in wormbase.org
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	Yes, we do deposition.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No