nature portfolio

Peer Review File

Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

Comments to the author:
In the manuscript "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy" the author explains about an interesting phenotype, apathy-like state in C. elegans with constitutive activation of SKN-1 transcription factor. The study goes on to address the involvement of key sensory signalling players like serotonin signalling in apathy-like behaviours. The work is culmination of several behavioural analyses, microscopic studies, RNA Sequencing studies and RNAi approaches. The study seems important considering the aspect of how apathy is similar to depression state in humans and the role of serotonin signalling highlights the role of SSRIs in such conditions. Though the work is well explained and presented, there are a few queries pertaining to it, which are needed to be addressed.

Major comments:
1. The author has stated WT animals move from the PA14 region to the E. coli during later hours. Has the author checked if it was due to depleted food source at the region upon which the animals move away?
2. Can the author rationalize why only SKN-1 was considered for the study? Has the author checked for other players which could be involved in such pathogenic avoidance behaviour?
3. Has the author checked the survivability of the animals which exhibit apathetic phenotype in PA14?
4. Line: 171; The attraction or the repulsion of the animal towards a food source is majorly due to sensing the odorants released or present in the food source. The author states that the apathetic food leaving behaviour is not associated with the perception of volatile odorants within the diet. In that case, can the author explain how the animal could have shown the modulated behaviour due to the food source?
5. Can the author explain how exactly intestinal bloating can alter the pathogen avoidance behaviour?
6. Acetylcholine has been vastly studied for their involvement in learning and memory in C. elegans. Is it possible for the author to check for cholinergic signalling along with the studied serotonin signalling?
7. Whether the author checked the same hypothesis with a loss of function mutant of SKN-1?
8. Since the study deals with serotonin signalling and SKN-1, can the author explain how these two players are correlated?
9. Since SKN-1 is a transcription factor, whether the author checked for modulations of the downstream players?
10. Whether the author validated the leads from the RNA Seq data with Real Time PCR based gene expression studies?

Reviewer #2 (Remarks to the Author):

The authors investigated the effects of SKN-1 constitutive expression in the maintenance of serotonin levels and behavioral immunity. Using C. elegans and Pseudomonas infection model they studied the role of neuronal and intestinal SKN-1 in the pathogen apathy behavior; they employed behavioral assay, genetics, RNAi, RNAseq techniques to address their queries. While the study has merits and may ignite interest among researchers in the field, however, some issues need to be addressed. Please find the attached annotated .pdf file.

Reviewer #3 (Remarks to the Author):

In the manuscript titled "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy," the authors explore the manifestation of pathogen apathy-like behavior in skn-1 gain-of-functon(skn-1 gf) mutants following exposure to the pathogenic bacteria PA14. Through targeted degradation of SKN-1 gf in specific tissues, they establish the necessity of neuronal SKN-1 gf activity for the observed pathogenic apathy. Additionally, differential expression of genes involved in serotonin signaling in response to skn-1 gf and PA14 infection suggests a potential link between serotonin deficiency and pathogen apathy. The authors demonstrate the restoration of pathogen apathy in skn-1 gf mutants upon serotonin supplementation, and further validate their findings by showing that mutations in tph-1 and pah-1, involved in serotonin biosynthesis, replicate the pathogen apathy phenotype.

This work sheds light on behavior in C. elegans, termed pathogen apathy, in response to infection, and elucidates underlying endocrine signaling by linking SKN-1 activity and serotonin. The characterization of skn-1 gf is commendable, reflecting the expertise of the Curran lab. However, the paper's scope and the extent of its advancement are somewhat narrow, suggesting a more suitable fit for a specialized journal. Specific concerns are outlined below:

Major concerns:
1. While the authors conduct extensive RNA seq experiments under pathogenic conditions and in skn-1 gf mutants, the analysis in Fig. 4 lacks clarity on why serotonin signaling was chosen for further investigation. The rationale behind the selection of serotonin signaling pathways should be elucidated. I also suggest performing qRT-PCR to measure tph-1 and pah-1 mRNA levels in skn-1 gf mutants under PA14 infection conditions. Additionally, genetic epistasis analysis between skn-1 gf and tph-1 or pah-1 should be conducted to further explore the relationship between SKN-1 activity and serotonin in the context of pathogen apathy.
2. The successful reversal of pathogen apathy in skn-1 gf mutants through serotonin supplementation is noteworthy. However, the mechanism by which pre-infection serotonin administration restores pathogen apathy requires elucidation. Exploring the effects of serotonin treatment post-PA14 infection or simultaneous treatment would provide valuable insights. Given previous research indicating serotonin's role in modulating SKN-1 localization in response to infection, it is crucial to investigate whether serotonin treatment affects SKN-1 localization in the context of PA14 infection. Addressing these concerns would strengthen the paper's conclusions.

Minor concerns
1. The graphical scheme of the food choice assay in Fig. 1A provides comprehensive information about assay conditions for each figure. However, I suggest labeling the conditions of experiments individually for clarity.
2. In Figure 2, the order of the figure legend for panels E, F, and G appears to have been altered. This should be corrected for consistency.
3. Please consider adding explanations for the arrows in Figure 3A, B, C, and D to enhance clarity. Additionally, including scale bars in these panels and detailing the quantification method for Figure 3E, F would improve the comprehensibility of the results.
4. It would be beneficial to include information on the roles of tph-1 and pah-1 in serotonin signaling in Figure 5B to provide additional context for readers.

We greatly appreciate all of the suggestions to improve our study. We have completed everything requested by the reviewers and the editors which has resulted in new data that can be found in the resubmission as indicated in each response below (indicated by arrowheads and *italicized text*).

Reviewer 1:
Comments to the author:

In the manuscript "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy" the author explains about an interesting phenotype, apathy-like state in C. elegans with constitutive activation of SKN-1 transcription factor. The study goes on to address the involvement of key sensory signalling players like serotonin signalling in apathy-like behaviours. The work is culmination of several behavioural analyses, microscopic studies, RNA Sequencing studies and RNAi approaches. The study seems important considering the aspect of how apathy is similar to depression state in humans and the role of serotonin signalling highlights the role of SSRIs in such conditions. Though the work is well explained and presented, there are a few queries pertaining to it, which are needed to be addressed.

➢ *We appreciate the reviewers positive assessment of our manuscript and have addressed each of the concerns raised below.*

Major comments:

1. The author has stated WT animals move from the PA14 region to the E. coli during later hours. Has the author checked if it was due to depleted food source at the region upon which the animals move away?

➢ *The Food choice assay plates were examined at various time intervals of the assay. No depletion of either diets was observed. To answer the query of the reviewer, pictures of the assay plate at these time points are now included in **Figure S1K**.*

2. Can the author rationalize why only SKN-1 was considered for the study? Has the author checked for other players which could be involved in such pathogenic avoidance behaviour?

➢ *Our lab focuses on skn-1gf mutants and the associated physiological effects of constitutive activation. We observed the pathogen apathy phenotype of our skn-1gf mutants, and this manuscript describes the regulation of this behavior. Although an exhaustive check of other pathways is beyond the scope of this manuscript, we now include a test of two longevity mutants, daf-2 and eat-2, but neither displayed the apathy phenotype observed in skn-1gf mutants. This new data is found in **Figure S1N-O**.*

3. Has the author checked the survivability of the animals which exhibit apathetic phenotype in PA14?

➢ *Yes, fast kill assays of wildtype and skn-1 gf were performed in **Figure 6A** and our previous work has demonstrated that skn-1gf mutant worms also display reduced lifespan in comparison to WT[1-3].*

4. Line: 171; The attraction or the repulsion of the animal towards a food source is majorly due to sensing the odorants released or present in the food source. The author states that the apathetic food leaving behaviour is not associated with the perception of volatile odorants within the diet. In that case, can the author explain how the animal could have shown the modulated behaviour due to the food source?

➢ *This is a great question and an important point of our story. Initial attraction/avoidance behaviors are unperturbed in skn-1gf mutant worms as animals reach food at the same time (**Figure S1G**). This is supported*

*by an overall similar response to chemotaxis shown in **Figures 2A &S2A-B** albeit the repellent 1-undecene[5] was not as strong in skn-1gf worms; but again, this difference is not sufficient to prevent animals from reaching the PA14 diet at the same time as WT. What is different in skn-1gf mutant worms is the leaving response from pathogen, which is known to be governed by intestinal distension[6], however, the bloating response in skn-1gf worms was found to be similar to WT worms **(Figure S3Q-T)**. Our results demonstrate that the failure to leave observed in skn-1gf mutant worms is due to lack of serotonin bioavailability.*

5. Can the author explain how exactly intestinal bloating can alter the pathogen avoidance behaviour?

➢ *This phenotype was previously described[6]. In brief, bloating of the intestinal lumen due to microbial colonization may be perceived as a danger signal that activates an immune fight-and-flight response via neuroendocrine signaling, however, we demonstrate is not the cause of apathy in skn-1gf mutant worms.*

6. Acetylcholine has been vastly studied for their involvement in learning and memory in C. elegans. Is it possible for the author to check for cholinergic signalling along with the studied serotonin signalling?

➢ *This is a great suggestion, which we include as new data. Acetylcholine supplementation did not alleviate the apathy response in skn-1gf mutants. This new data in **Figure S5E** further supports our model that the apathy response is specific to defects in serotonin bioavailability.*

7. Whether the author checked the same hypothesis with a loss of function mutant of SKN-1?

➢ *The apathy response is due to SKN-1 activation (either by skn-1gf allele or loss of the negative regulator wdr-23, **Figure 1F**). However, as requested, we now include an examination of the skn-1lf mutant skn-1(ok2315) which does not display any apathy phenotype (**Figure S1P**).*

8. Since the study deals with serotonin signalling and SKN-1, can the author explain how these two players are correlated?

➢ *Our examination of serotonin stemmed from our examination of the transcriptional responses governed by SKN-1. Genes associated with serotonin signaling were prominent in the context specific responses to OP50 and PA14. We now include data that demonstrated serotonin levels are critically low in skn-1gf mutants (**Figure 5D**), which was also validated via fluoxetine sensitivity assay (**Figure 5E**). Although RNAseq is more sensitive, we confirmed the expression of serotonin pathway genes via RT PCR (**Figure S5G-H**) and found tph-1 levels are indeed reduced in WT and skn-1 gf mutants upon PA14 exposure. Moreover, pah-1 is reduced in WT upon pathogen exposure but is constitutively lower in skn-1gf mutants.*

9. Since SKN-1 is a transcription factor, whether the author checked for modulations of the downstream players?

➢ *Indeed, the context-specific RNAseq data (WT, skn-1gf, OP50, PA14) is included in this manuscript in **Figures 4 and S4**. The raw data is also available to the community in the NIH Gene Expression Omnibus (GEO; GSE251677).*

10. Whether the author validated the leads from the RNA Seq data with Real Time PCR based gene expression studies?

➢ *RT-qpCR is less sensitive than RNAseq; qPCR is more prone to normalizing biases due to the choice of control genes which may vary themselves versus RNA-seq which performs unbiased normalization at the transcriptome-wide level[8,9]. Nevertheless, we have validated the expression of the two key serotonin biosynthesis pathway genes in this study by qPCR which confirmed the RNAseq data (**FigureS5G-H**).*

Reviewer #2 (Remarks to the Author):

The authors investigated the effects of SKN-1 constitutive expression in the maintenance of serotonin levels and behavioral immunity. Using C. elegans and Pseudomonas infection model they studied the role of neuronal and intestinal SKN-1 in the pathogen apathy behavior; they employed behavioral assay, genetics, RNAi, RNAseq techniques to address their queries. While the study has merits and may ignite interest among researchers in the field, however, some issues need to be addressed. Please find the attached annotated .pdf file.

*1. **Line 58-61**:"Pseudomonas aeruginosa (PA14) is a human opportunistic pathogen that is lethal to C. elegans with prolonged exposure. The p38 mitogen activated protein kinase (MAPK) pathway and many of the downstream transcriptional effectors are evolutionarily conserved, indicating a critical role potentiating cellular responses"*

Reviewer comment: Please revise this paragraph. Connect the opening sentence with the rest of the sentences in the paragraph.

- *We have edited the manuscript as suggested.*

*2. **Line 69-70** "Among the physiological consequences of PA14 exposure is the rapid depletion of stored intracellular lipids in the intestine;"*

Reviewer comment: Please check the flow of the sentences. The PA14 literatures may follow the opening statement of the previous paragraph?

- *We have edited the manuscript as suggested.*

*3. **Line 96** "[1]. The ability to avoid pathogenic bacteria, even after a brief exposure, is essential for the health"*

Reviewer comment: "please check the formatting"

- *We have edited the manuscript as suggested.*

*4. **Line 119 :** "(Figure 1A and Table S1)"*

Reviewer comment: "Please provide Table S1. The current link for Table S1 contains supplementary dataset, not the actual supplemental table. Also please check the manuscript item list. There are two Table S1s. "Table S1 Supplementary Dataset (154KB) Source File (XLSX) 154KB Table S1 Supplementary Dataset (13KB) Source File (XLSX) 13KB"

- *The main manuscript with the corresponding source data table and supplementary tables and datasets have been uploaded with the current version of the manuscript.*

*5. **Line 125:** "most dynamic leaving response when the PA14 pathogen was cultured at 37°C"*

Reviewer comment: "Please define the word "most dynamic leaving response". Based on Fig. S1 animals subjected to 24h or 48h cultures didn't make any choice differences across all time points- 30m to 4h."

- *We have edited the manuscript as suggested.*

*6. **Line 126:** (Figure S1A).*

Reviewer comment: "Please note that the figures, labels, legends are extremely small in the current version."

➢ *We thank the reviewer for pointing out this oversight on our part. We have adjusted the figures to conform with the guidelines of the journal.*

7. ***Line 156:*** *"Pathogen apathy is a specific neuronal response"*

Reviewer comment: Which specific neuron or neuron pairs is suggested here in the sub-heading? As ASI neurons were not involved, Line 178. Please revise the sub-heading.

➢ *We apologize for the confusion. We have edited the manuscript to make it clearer that constitutive SKN-1 activation in the nervous system is necessary, but alone is not sufficient to induce pathogen apathy.*

8. ***Line 178:*** *"skn-1a"*

Reviewer comment:"To improve the ease of reading, please include the relevant genotypes of each strain of C. elegans, ideally only at the first mention? For example skn-1a (XYZ, Pga-4:skn-1gf(a))."

➢ *Based on the reviewers suggestions edits have been made in the lines: 1) 181: "Surprisingly, the expression of* ***skn-1a (Pga-4:skn-1gf(a)) or skn-1c (Pga-4:skn-1gf(c))*** *in the ASI could not replicate the failed pathogen leaving behavior observed in the skn-1gf mutant neurons (Figure 2E-F)." 2) 235-237: "Moreover, intestinal-specific co-expression of the gain-of-function variant of skn-1a/c i.e.* ***vha-6:skn-1gf(a&c)*** *(****Figure 3H****), nor the expression of the individual gain-of-function variants of skn-1a (****vha-6:skn-1gf(a****) or skn-1c (****vha-6:skn-1gf(c****) (****Figure S3K-3L****)"where the relevant genotypes of each strain of C. elegans, have been included at the first mention.*

9. ***Line 179:*** *"leaving"*

Reviewer comment: "For clarity, please us pathogen 'apathy' behavior? It is understood that "pathogen leaving behavior' phenomenon is described here, but for ease of reading and understanding it may be better to describe the scenario- leaving or apathy. Please check elsewhere in the MS as well."

➢ *The mentioned edits have been taken into consideration and where so possible we have tried to maintain consistency with usage of the term "apathy" throughout the manuscript.*

10. ***Line 184:*** *"with loss of SKN-1wt-AID"*

Reviewer comment: "Please clarify. Is the loss of SKN-1-AID gene construct induced? or It is to describe WT control 'without' the gene construct?"

➢ *We have clarified this section and include relevant references to the methodologies. For this specific query, the loss here is the tissue-specific degradation of SKN-1wt AID driven by treatment with auxin.*

11. ***Line 187:*** *"pan-neuronal expression"*

Reviewer comment: "Is it known that the level of expressions driven by the promoters Pgpa-4, Pvha-6, pan-neuronal promoters are comparable to the Pskn-1?"

➢ *This is an important point and we have included a discussion of this point in the discussion. The endogenous expression of skn-1 is lower in each of these tissues/cell types (Packer et al 2019 and CeNGEN; data available on Wormbase) as compared to the expression induced by these tissue specific drivers; as such the constitutive SKN-1 activity that induces pathogen apathy is likely stronger in these individual strain (but*

restricted to distinct tissues) and the expression of TIR1 for the auxin-mediated degradation is more strongly expressed in these tissues under these drivers. See ***Table R1*** *(from Wormbase) below for this comparison.*

	Intestine	***ASI neurons***	***Whole animal***
Read counts of tissue-specific driver	***vha-6 promoter*** ASI neurons – 5.2 TPM intestine anterior – 864.37 TPM intestine middle - 531.29 TPM intestine posterior – 549.64 TPM	***gpa-4 promoter*** ASI neurons - 413 TPM intestine anterior – ND intestine middle - ND intestine posterior – ND	***skn-1 promoter*** ASI neurons – 44 TPM intestine anterior – 97.37 TPM intestine middle - 49.1 TPM intestine posterior – 68.38 TPM
Table R1. Transcripts Per Million (TPM); Single cell gene expression graphs in Wormbase – CeNGEN and Packer et al 2019; ND = not detected			

12. ***Line 200:*** *"CRISPR/CAS9 edited"*

Reviewer comment: "Please clarify this statement. Was a GFP variant used in the experiment? If yes, please provide the rationale. Is it necessary to include the CRISPR mediated editing details here? or otherwise it may be omitted?"

➢ *We provided the details of each strain used for the intestinal localization experiment upon its first mention in the manuscript and also in the methods section; but in brief, GFP was engineered to create a translational fusion to the endogenous locus, but this strain is only used for the localization studies, all physiology and behavior is done without the GFP fusion.*

13. ***Line 202:*** *"intestine. Outside the constitutive expression of both SKN-"*

Reviewer comment: "This statement is confusing. Is the exposure exclusive to the intestinal cells, and the ASI neurons are isolated?"

➢ *Related to point 12 above, yes, SKN-1 is constitutively expressed in the ASI neurons (with GFP expression observed in the ASI neuron pair) irrespective of pathogen (PA14) exposure or not. In addition, upon PA14 exposure we see intestinal nuclear localization within a time period of 10-40 mins with the help of the reporter strains SKN-1wt-GFP and SKN-1-gf-GFP.*

14. ***Line 204:*** *"(Figure 3A-E,"*

Reviewer comment: "Please provide relevant scale bar and magnification."

➢ *The relevant scale bar and magnification of* ***Figure 3A-E*** *have been uploaded in the current version of the manuscript. The microscopy details have been provided in the "Imaging" subsection of the "MATERIAL AND METHODS" section of the manuscript.*

15. ***Lines 204-205:*** *"(Table S2,"*

Reviewer comment: "Please recheck Table S2 link."

➢ *The main manuscript with the corresponding source data table and supplementary tables and datasets have been uploaded with the current version of the manuscript.*

16. ***Lines 207-208:*** *"data suggest that the failure to leave a pathogenic environment is not a result of failed stabilization and subcellular localization of SKN-1."*

Reviewer comment: "This conclusion is misleading. The experiments that are performed did not measure the stabilization of the protein. For example, one may look the duration of fluorescence of fused-GFP, SKN-1 turn-over or duration of nuclear localization.

- *Based on the reviewers suggestions to look into the duration of fluorescence of fused-GFP, SKN-1 turn-over; We now include our measurements of the loss of SKN-1 nuclear localization post PA14 exposure. No major changes were observed in SKN-1 localization pattern post PA14 exposure between WT and skn-1gf worms (**Figure S3G-P)**.*

Also based on the observations, the behavior observed was in fact due to sub-cellular localization. i.e. accelerated and increased abundance of sub cellular SKN-1 at this point. Only experiments performed below with the intestinal specific approach debunked that these observations are corelated effects and not causative."

- *We note that the changes in timing and abundance of SKN-1gf protein in the intestine is modest at best. The fact that tissue specific expression of SKN-1gf in the intestine could not induce apathy and the tissue specific degradation could not reverse it. As the reviewer notes, the apathy behavior observed in skn-1gf worms and the SKN-1 protein dynamics might be correlated but is clearly not be causative.*

*18. **Lines 218-219**: "unlike the nervous system where SKN-1gf is required for pathogen apathy behaviors,"*

Reviewer comment: "How is the conclusion drawn, that the nervous system was required for constitutive SKN-1gf mediated apathy; if the expression of pan-neuronal Prgef-:SKN-1gf expression did not phenocopy the apathy behavior? The apathy observed due to auxin mediated degradation of paneuronal SKN-1gf would suggest that SKN-1gf was active elsewhere, and of course not in the intestinal cells, as degradation or expresion here did not resulted in leaving/apathy. Please reconcile the observations. It is also entirely possible that the reviewer is not fully understanding the different intertwining scenarios. In that case provide a better cartoon model to help better understanding."

- *We have edited the text to make this clearer. Because loss of neuronal SKN-1gf reversed the apathy behavior (**Figure 2G**), we conclude that SKN-1 activity in neurons is required. This is opposed to loss of SKN-1gf expression in the intestine which did not change pathogen apathy (**Figure 3G**). Nevertheless, we now include new data where we co-express skn-1gf in both neurons and the intestine which can partially induce pathogen apathy behaviors (**Figure 3I**) while expression only in the intestine did not (**Figure 3H**). With this we conclude that neurons expression alone is necessary but not sufficient and intestine expression alone is neither necessary nor sufficient .*

*19. **Lines 239-240**: "The sustained intestinal distension in response to PA14 exposure in animals with the skn-1gf allele"*

Reviewer comment: "Please revise the introductory statement.

Please provide data related to sustained intestinal distention in skn-1gf animals as on line 224 and 225, it is mentioned that "the bloating was unremarkable". Further, previous authors [19] observed increased intestinal distension and increase lawn avoidance, while skn-1gf animals had sustained intestinal distension and no enhancement of avoidance? Additionally, lawn occupancy/avoidance and binary choice experiments are quite different experimentally. It may be hard to have a direct comparison between behaviors observed on these different experiments operating very differently."

- *We have edited the manuscript as suggested.*
- *The unremarkable difference in intestinal distension between WT and skn-1gf is found in **Figure S3Q-T***

➢ *The reviewer has identified the key observation of our manuscript that despite intestinal distension, the skn-1gf mutants on pathogen fail to leave the PA14 environment. We further show that this lack of pathogen leaving behavior is due to limited serotonin.*

20. ***Lines 334-335:*** *"Taken together, these data reveal a consequence of constitutive SKN-1 activation is the depletion of available serotonin"*

Reviewer comment: "These data may not directly prove that SKN-1 constitutive expression reduces serotonin reserve which is the main claim and the title of the MS. The only bridge that is presented so far is the transcript level differential expression of serotonin synthesis gene. No doubt serotonin depletion messed the pathogen avoidance behavior. Serotonin levels may be measured in the body/lysate of WT and skn-1gf animals?"

➢ *We thank the reviewer and now report our measurements of serotonin levels in WT and skn-1gf mutants via Serotonin ELISA and found the serotonin levels to be remarkably low in skn-1gf mutants (**Figure 5D**). We also checked the expression of serotonin pathway genes via RT PCR and found tph-1 levels low in WT and skn-1 gf mutants upon PA14 exposure and pah-1 to be low in WT upon pathogen exposure and constitutively low in skn-1gf mutants (**Figure S5G-H**). Moreover, a consequence of low serotonin in skn-1gf mutants is an enhancement of toxicity to the selective serotonin reuptake inhibitor fluoxetine (**Figure 5E**). Taken together these data collective support the model where serotonin is limiting in skn-1gf mutants.*

21. ***Figure 1.*** *Activation of SKN-1 drives pathogen apathy.*

Reviewer comment: Figure 1A " These legends doesnt belong here?"
Figure 1C "The labels, legends are extremely small at the current resolution, please note."
Figure 1D "It is confusing in the current form. Cartoon diagram for PA14 (brown line) may also be included?"
Line 423: skn-1gf "italics"

➢ *We have edited the manuscript as suggested.*

22. ***Figure 3.*** Precocious activation of intestinal SKN-1 in responses to PA14***.***

Reviewer comment: Figure 3A-D "Please include scale bars and magnification factors."

➢ *The suggested changes of including scale bars and magnification factors have been included in the current version of the manuscript.*

23. ***Figure 4.*** Context dependent transcriptional signature of PA14 exposure.
Reviewer comment: Figure 4C "illegible. Please revise."
➢ *Thank you for pointing this out. All figure fonts have been revised to the journal standards*

24. ***Figure 5.*** Pathogen apathy is a serotonin signaling defect.
Reviewer comment: Figure 5A "illegible."

➢ *Thank you for pointing this out. All figure fonts have been revised to the journal standards*

25. ***Supplemental Figure S3.*** Nuclear accumulation of SKN-1 and intestinal bloating in response to PA14..

Reviewer comment: Supplemental Figure S3 A-F "Please include scale bars and magnification factors."

➢ *The suggested changes of including scale bars and magnification factors have been included in the current version of the manuscript.*

26. ***Supplemental Figure S5.*** Pathogen apathy with constitutive SKN-1 is associated exclusively with serotonin.

Reviewer comment: Supplemental Figure S5 A "Very hard to read at the current resolution."

- *Thank you for pointing this out. All figure fonts have been revised to the journal standards.*

27. ***Lines 334-335:*** *"SUPPLEMENTAL TABLES"*

Reviewer comment: "Are the tables related to RAW supplemental data or separate data? Please check."

- *The main manuscript with the revised corresponding source data table and supplementary tables and datasets have been uploaded with the current version of the manuscript. Moreover, the RNAseq data is available on GEO.*

All line numbers in the "Reviewer 2" comments section is to be referred to the previous submitted manuscript version. The corresponding edited current version may not be having the same line numbers.

Reviewer #3 (Remarks to the Author):

In the manuscript titled "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy," the authors explore the manifestation of pathogen apathy-like behavior in skn-1 gain-of-functon(skn-1 gf) mutants following exposure to the pathogenic bacteria PA14. Through targeted degradation of SKN-1 gf in specific tissues, they establish the necessity of neuronal SKN-1 gf activity for the observed pathogenic apathy. Additionally, differential expression of genes involved in serotonin signaling in response to skn-1 gf and PA14 infection suggests a potential link between serotonin deficiency and pathogen apathy. The authors demonstrate the restoration of pathogen apathy in skn-1 gf mutants upon serotonin supplementation, and further validate their findings by showing that mutations in tph-1 and pah-1, involved in serotonin biosynthesis, replicate the pathogen apathy phenotype.

This work sheds light on behavior in C. elegans, termed pathogen apathy, in response to infection, and elucidates underlying endocrine signaling by linking SKN-1 activity and serotonin. The characterization of skn-1 gf is commendable, reflecting the expertise of the Curran lab. However, the paper's scope and the extent of its advancement are somewhat narrow, suggesting a more suitable fit for a specialized journal. Specific concerns are outlined below:

Major concerns:

1. While the authors conduct extensive RNA seq experiments under pathogenic conditions and in skn-1 gf mutants, the analysis in Fig. 4 lacks clarity on why serotonin signaling was chosen for further investigation. The rationale behind the selection of serotonin signaling pathways should be elucidated.

- *Upon looking into the RNA sequencing data of WT and skn-1gf mutants with and without PA14 exposure we found differentials in the expression of serotonin pathway genes, further we found serotonin levels to be low in skn-1gf mutants (**Figure 5D**), which was also validated via fluoxetine sensitivity assay (**Figure 5E**).*
- *Finally, we tested supplementation of several signaling molecules like dopamine, octopamine as provided in (**Figure S5B-E)** as well as new data with acetylcholine (**Figure S5E**) and serotonin was the only signaling molecule that altered the apathy response in skn-1gf (**Figure 5F**).*

I also suggest performing qRT-PCR to measure tph-1 and pah-1 mRNA levels in skn-1 gf mutants under PA14 infection conditions.

- *Although RNAseq is more sensitive, as requested we confirmed the expression of serotonin pathway genes via RT PCR (**Figure S5G-H**) and found tph-1 levels are indeed reduced in WT and skn-1 gf mutants upon PA14 exposure. Moreover, pah-1 is reduced in WT upon pathogen exposure but is constitutively lower in skn-1gf mutants.*

Additionally, genetic epistasis analysis between skn-1 gf and tph-1 or pah-1 should be conducted to further explore the relationship between SKN-1 activity and serotonin in the context of pathogen apathy.

- *Since skn-1gf and the tph-1lf pah-1lf mutants have both low serotonin and each display pathogen apathy we cannot perform genetic epistasis analysis as the directionality is the same. However, we include data that the pathogen apathy behavior in the tph-1lf pah-1lf also reversed by serotonin supplementation (**Figure 5F & 6D**) in both mutant backgrounds, indicating that the behavior is related to the low serotonin state in both. Moreover, although less satisfying, we attempted to make the skn-1gf; tph-1lf pah-1lf triple mutant, but after genotyping more than 200 progeny from animals homozygous for skn-1gf and heterozygous for tph-1lf pah-1lf, we were unable to recover an animal homozygous for tph-1lf pah-1lf (noting these two genes are tightly linked). We are following up on this synthetic lethality, but believe this analysis is beyond the scope of this work.*

2*. The successful reversal of pathogen apathy in skn-1 gf mutants through serotonin supplementation*

is noteworthy. However, the mechanism by which pre-infection serotonin administration restores pathogen apathy requires elucidation.

➢ *Based on the reviewers concern, we did look into SKN-1 localization (with the help of SKN-1$_{wt}$:GFP and SKN-1$_{gf}$:GFP) when treated with serotonin prior to PA14 exposure. No major changes were observed in SKN-1 localization pattern upon PA14 exposure between the treated and untreated serotonin populations (i.e. WT vs WT+Serotonin and skn-1gf vs skn-1gf +Serotonin) (**Figure S6A-F**).*

Exploring the effects of serotonin treatment post-PA14 infection or simultaneous treatment would provide valuable insights. Given previous research indicating serotonin's role in modulating SKN-1 localization in response to infection.
Temporal serotonin treatment data it is crucial to investigate whether serotonin treatment affects SKN-1 localization in the context of PA14 infection. Addressing these concerns would strengthen the paper's conclusions.

➢ *Further we examined the loss of SKN-1 nuclear localization when treated with serotonin all throughout i.e. prior and post PA14 exposure. No major changes were observed in SKN-1 localization pattern post PA14 exposure between the treated and untreated serotonin populations (i.e. WT vs WT+Serotonin and skn-1gf vs skn-1gf +Serotonin) (**Figure S6G-L**). Moreover, we note the importance of pre-treatment of serotonin from the L1 stage as starting treatment at the **L3** stage was not sufficient to reverse pathogen apathy (**Figure 5G**).*

Minor concerns

1. The graphical scheme of the food choice assay in Fig. 1A provides comprehensive information about assay conditions for each figure. However, I suggest labeling the conditions of experiments individually for clarity.

➢ *We have edited the manuscript as suggested.*

2. In Figure 2, the order of the figure legend for panels E, F, and G appears to have been altered. This should be corrected for consistency.

➢ *Thank you identifying this issue. We have edited the manuscript as suggested.*

3. Please consider adding explanations for the arrows in Figure 3A, B, C, and D to enhance clarity. Additionally, including scale bars in these panels and detailing the quantification method for Figure 3E, F would improve the comprehensibility of the results.

➢ *For the explanation of arrows the following statement: "The first two intestinal nuclei of each worm are outlined by white-dotted circles and the constitutive expression SKN-1 in the ASI neurons is marked with white arrows." Has been incorporated in the legends of **Figure 3**, **Supplementary Figure 3** and in **Figure S6**. Each of the panels with scale bar has been provided in the current uploaded version of the manuscript. Further for the comprehensibility of the result the details of the quantification method the following statement: "For the population studies the a total of 60 (N=3;n=20) animals were counted per time point per condition. For quantification studies a minimum of three animals per time point per condition was taken into consideration with three biological replicates (N=3)", has been incorporated in the legends of **Figure 3, Supplementary Figure S3,** and in **Figure S6.***

4. It would be beneficial to include information on the roles of tph-1 and pah-1 in serotonin signaling in Figure 5B to provide additional context for readers.

- *According to the reviewer's suggestion information on the roles of tph-1 and pah-1 in serotonin signaling has been included in the manuscript. The following statements: 1) "In C. elegans the tph-1 gene encodes a tryptophan hydroxylase that catalyzes the first and rate-limiting step of neuronal serotonin biosynthesis[10,11]"; 2) "Recently, pah-1 encoding a phenylalanine hydroxylase has been identified to play key role in the non-canonical non-neuronal synthesis of serotonin" has been incorporated in the have been incorporated in the result section.*

1. Pang S, Curran SP. Adaptive capacity to bacterial diet modulates aging in C. elegans. *Cell Metab.* 2014;19(2):221-231.
2. Paek J, Lo JY, Narasimhan SD, et al. Mitochondrial SKN-1/Nrf mediates a conserved starvation response. *Cell Metab.* 2012;16(4):526-537.
3. Nhan JD, Turner CD, Anderson SM, et al. Redirection of SKN-1 abates the negative metabolic outcomes of a perceived pathogen infection. *Proc Natl Acad Sci U S A.* 2019;116(44):22322-22330.
4. Prakash D, Siddiqui R, Chalasani SH, Singh V. Pyrrole produced by Pseudomonas aeruginosa influences olfactory food choice of Caenorhabditis elegans. *bioRxiv.* 2022:2022.2001.2027.477966.
5. Prakash D, Ms A, Radhika B, Venkatesan R, Chalasani SH, Singh V. 1-Undecene from Pseudomonas aeruginosa is an olfactory signal for flight-or-fight response in Caenorhabditis elegans. *Embo j.* 2021;40(13):e106938.
6. Singh J, Aballay A. Microbial Colonization Activates an Immune Fight-and-Flight Response via Neuroendocrine Signaling. *Dev Cell.* 2019;49(1):89-99.e84.
7. Gusarov I, Shamovsky I, Pani B, et al. Dietary thiols accelerate aging of C. elegans. *Nature Communications.* 2021;12(1):4336.
8. Garrido-Gomez T, Castillo-Marco N, Clemente-Ciscar M, et al. Disrupted PGR-B and ESR1 signaling underlies defective decidualization linked to severe preeclampsia. *eLife.* 2021;10:e70753.
9. Singh PP, Benayoun BA. Considerations for reproducible omics in aging research. *Nat Aging.* 2023;3(8):921-930.
10. Sze JY, Victor M, Loer C, Shi Y, Ruvkun G. Food and metabolic signalling defects in a Caenorhabditis elegans serotonin-synthesis mutant. *Nature.* 2000;403(6769):560-564.
11. Sze JY, Zhang S, Li J, Ruvkun G. The C. elegans POU-domain transcription factor UNC-86 regulates the tph-1 tryptophan hydroxylase gene and neurite outgrowth in specific serotonergic neurons. *Development.* 2002;129(16):3901-3911.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised manuscript "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy" the authors have answered all the queries raised. The authors have also provided new data in order to answer the questions.

Reviewer #2 (Remarks to the Author):

The authors have addressed all the technical queries by conducting new experiments and providing better explanations. The manuscript is thoroughly improved, job well done!

Typographical error: Please check references 17 & 20, they are repeated.

Reviewer #3 (Remarks to the Author):

Although I still believe this manuscript fits a more specialized journal, I am okay with the decision by the editor giving the opportunity ofr revising the manuscript. The authors addressed the majority of my concerns. I have some remaining concerns as follows.

Regarding the qPCR results shown in Figure S5G-H, although the pah-1 mRNA levels were reduced by skn-1 gf under OP50 condition, the skn-1 gf did not alter the expression of pah-1 or tph-1. How could the authors claim that skn-1 gf drive pathogen apathy by altering the expression of these genes? I am conduesed because the expression levels of pah-1 or tph-1 were comparable in the WT PA14 and the skn-1 gf PA14 conditions. This should be clarified.
For the epistasis analysis between skn-1 gf and tph-1/pah-1, they can perform RNAi if they have issues with linkage.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

In the revised manuscript "Serotonin deficiency from constitutive SKN-1 activation drives pathogen apathy" the authors have answered all the queries raised. The authors have also provided new data in order to answer the questions.

RESPONSE: We thank the reviewer for the positive assessment of our work.

Reviewer #2 (Remarks to the Author):

The authors have addressed all the technical queries by conducting new experiments and providing better explanations. The manuscript is thoroughly improved, job well done!

Typographical error: Please check references 17 & 20, they are repeated.

RESPONSE: We appreciate the reviewer's help that has improved our manuscript. Thank you for catching the duplicated reference. We have edited the manuscript accordingly.

Reviewer #3 (Remarks to the Author):

Although I still believe this manuscript fits a more specialized journal, I am okay with the decision by the editor giving the opportunity ofr revising the manuscript. The authors addressed the majority of my concerns. I have some remaining concerns as follows.

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RESPONSE: We are sorry this is confusing. At <u>no point</u> are we suggesting that SKN-1gf is directly regulating the expression of either pah-1 or tph-1. In fact, it is highly unlikely that this is the case as skn-1gf is a constitutively activated transcription factor that results in the INCREASE expression of target genes and pah-1 levels are reduced. Several serotonin-related genes were altered in a context dependent manner between the two genotypes and bacterial diets. The connection to pah-1 and tph-1 is that skn-1gf mutants have reduced serotonin and PAH-1 and TPH-1 are the enzymes that generate serotonin.

For the epistasis analysis between skn-1 gf and tph-1/pah-1, they can perform RNAi if they have issues with linkage.

RESPONSE: The idea of using RNAi is great, but again the skn-1gf phenotype and tph-1lf pah-1lf phenotype are both in the same direction (each induce apathy individually) so we cannot elucidate an epistatic relationship (which mutation suppresses apathy). Nevertheless, we now include this data as requested in supplemental figure 5I. To induce SKN-1 activation we use wdr-23 RNAi, as previously shown in Figure 1F but here now, in the tph-1lf pha-1lf mutant background. This avoids the need for double RNAi (tph-1 and pah-1) and the issues with RNAi efficacy in neurons. As predicted, the apathy phenotype persists in tph-1lf pah-1lf mutants when SKN-1 is constitutively activated from loss of the negative regulator wdr-23.

REVIEWERS' COMMENTS

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The authors addressed my concerns.