nature portfolio

Peer Review File

A specific folate activates serotonergic neurons to control C. elegans behaviour

Reviewers' Comments:

Reviewer #1:
Remarks to the Author:
This study explores the relationship between folate vitamin related molecules, their interaction with the C. elegans FOLR-1 folate transporter, and potential activation of the TRPM-family channel GON-2 in the regulation of egg-laying and dwelling behaviors. The authors first show that FOLR-1 is expressed in two major serotonin-producing and releasing cells, the HSNs and NSMs, which release serotonin to promote egg-laying and dwelling, respectively. The 10F-THF and DHP folate derivatives promote egg laying in briefly starved wild-type animals but not mutant animals lacking the FOLR-1 receptor or the TRPM channel GON-2, both of which the authors show are co-expressed in NSM and HSN. As the induction of egg laying is relatively rapid, within ~10 minutes or so, the authors postulate these folate-related molecules promote egg laying via a signaling, not metabolic, pathway. This conclusion is supported by evidence that 5-methyl-THF, 5-formyl-THF, and THF did not have the same effect. While these metabolic vs. signaling questions are outside the expertise of this reviewer, I do not believe these experiments exclude a metabolic role for these small molecules, for example in biopterin-dependent serotonin biosynthesis that could contribute to the behavior results observed.

Because both folr-1 and gon-2 mutants are resistant to 10F-THF and DHP-induced egg laying, and that both genes contribute to germline development, the authors tested their hypothesis that these two membrane proteins interact physically. Using a transient over-expression approach in HEK293 cells, the authors detect co-immunoprecipitation from detergent extractions, suggesting they are part of a complex.
The authors then go on to show that THF and DHP (weakly) can stimulate HSN Ca2+ transient activity. The stimulation of egg laying by 10F-THF similarly depends upon serotonin and HSN function, as it is lost in serotonin-deficient tph-1 mutants or egl-1(dm) animals lacking HSNs. This data is consistent with HSN-released serotonin being required for the 10F-THF effect. The authors then try to determine where FOLR-1 is acting through cell & tissue-specific rescue experiments. Expression of FOLR-1 just in HSN and NSM fails to restore 10F-THF stimulation of egg laying, suggesting additional sites of action. Co-expression of FOLR-1 in the isthmus and intestinal-valve were required for full rescue which the authors interpret as showing additional roles for FOLR-1 in transport of 10F-THF for its egg-laying behavior effects. This result complicates interpretation of the previous HSN Ca2+ activity results, as the DHP and 10F-THF stimulatory effect and its reduction in folr-1 (and maybe gon-2) mutants may be upstream of HSN.

The authors then turn to NSM functions of folr-1 and gon-2 finding a very interesting observation that these mutants have a defect in enhanced-slowing after brief starvation and reencountering of food. The authors describe this in terms of recovery of locomotion after the initial slowing, but the videos suggest the worms may not slow that much in the first place.

Overall, there are some interesting data here, and the experiments are well-performed. The issue is the interpretation and how to bring these pieces together in a way that makes sense. Evidence for a direct and functional important interaction where folate activation of FOLR-1 facilitates TRPM

channel activity in HSN or other cells remains speculative. The HSNs still show robust Ca2+ activity in both mutants, although that does not preclude them functioning in some way to modulate cell excitability. The author's results showing FOLR-1 functions in several cells upstream of HSN suggests the Ca2+ effects seen may be indirect. I am concerned about the interpretation by the authors for a signaling vs. metabolic role since most of the data could be explained by changes in serotonin signaling for both egg-laying and slowing behaviors. A prediction would be that these mutants have serotonin deficiency and that exogenous DHP / 10F-THF helps elevate serotonin levels by promoting its synthesis. This could be tested directly through anti-serotonin antibody staining (Tanis et al. 2008) or other, more direct serotonin measurements. Perhaps one of their derivatives cannot contribute to serotonin biosynthesis. Such a result would not be unprecedented, as I think this is why folate and biopterin deficiencies are thought to contribute to depressive behavior. If the authors are convinced this model is unlikely, it would be helpful if they navigate that in the Discussion and/or Results. As it is, it does not seem to be addressed as a possibility even to rule out. Again, this is outside the metabolic expertise of this reviewer, but that may also be true for a generic reader of the paper who might be similarly curious about FOLR-1's role in serotonin biosynthesis.

Major points:
- Is there any prior evidence from other studies or systems that FOLR-1 and GON-2 would interact? If so, the authors should include such a connection because it is a bit of a leap. Why would such an interaction be expected and how would it facilitate either proteins biological functions? If the authors are really convinced the functions of these two proteins is integral, it may be worth speculating on its functional significance.
- The authors conclude from their co-immunoprecipitation experiments that FOLR-1 and GON-2 "interacted strongly with each other," but there is no quantitative evidence to support such a determination. The authors do not report the extent to which these co-expressed proteins are associated with each other. The interaction may also be indirect. The extent could be estimated from densitometric quantitation relative to how much protein was present in the starting lysate. The authors should report. either in the Materials & Methods or the Figure 3C legend itself, how much lysate was run and whether the exposures presented in different gel cutouts are comparable. Regardless, the authors should temper their conclusions about the relative strength of the interaction absent additional evidence. The proteins could just be sticky. If demonstrating such a direct interaction was critical to the authors, they could perform additional control experiments like performing the co-immunoprecipitation experiment from a mixed detergent lysate prepared from separate transgenic cells expressing only a single protein.
- In Figure 4a and 4b, it's unexpected that the egl-1(dm) mutant lays a similar number of eggs as the tph-1 mutant, considering they are much more Egl. I would expect them to be less likely to lay eggs under these conditions, certainly more defective than wild-type animals even though the number of eggs laid are not dramatically different. Admittedly, the M9-agarose assay used here is atypical and may lead to non-intuitive results. It seems (and the authors state this directly) that this assay does have some variability across experiments, so it would be useful to know if wild-type animals were assayed alongside the tph-1 and egl-1 mutant data shown here. If that data was collected at the same time, it should be shown.
- It is surprising that the gon-2/hT2 mutant is effective as a heterozygote. It would be helpful if the

authors could speculate in the Discussion why you feel this mutant has as strong phenotype as a het. They may not be able to get very far, although it is not unprecedented for signaling mutants that affect egg-laying behavior to have semi-dominant effects (although this reviewer is having difficulty finding published papers that report that fact directly).
- How were animals synchronized? The methods describe animals as having a single row of eggs, but that would likely vary among the genotypes used in this study (for example, egl-1(dm) mutants). Did the authors use other methods, for example staging from timed egg lays, bleached egg preps, or L4's? Please add that information.
- The data in 6b and 6c do not appear to be normally distributed. It may make more sense to use non-parametric statistical tests and focus on median vs. means.

Reviewer #2:
Remarks to the Author:
Review of Peesapati et al., "Folate activates serotonergic neurons to control C. elegans behaviour'.

The authors present an interesting story about how a specific folate that is food (bacteria)-derived impacts neuronal calcium activity and in turn, the behaviour of the nematode C. elegans. As far as the authors report (I am not an expert in folate biology), this appears to be the first evidence beyond expression patterns in mice of a folate receptor being involved in neuronal signalling, which is interesting. The authors provide substantial genetic evidence that the folate receptor (FOLR-1) functions in select neurons and tissues that may mediate folate transport. They also provide direct and circumstantial lines of evidence for FOLR-1 interaction with the GON-2 calcium channel. A mechanistic model is suggested (folate interaction with FOLR-1 modifies GON-2 channel activity) in the discussion, but not tested in a heterologous assay such as in Xenopus oocytes.

Comments

Eighth paragraph of the results (this would have been easier with line numbers in the manuscript): I am not convinced that one-carbon metabolism is not relevant to the phenotypes being studied based on this paragraph alone (which is what they conclude at the end of the paragraph). The authors report that only 10F-THF, but not other physiological folates (what is a physiological folate btw?), can modulate germ cell proliferation. They then go on to report that several folates, including 10F-THF can rescue elegans folate deficiency. Is a germ cell proliferation phenotype not part of folate deficiency? I find this confusing. It seems that some phenotypes are sensitive only to 10F-THF while others are not. It is not clear to me how this can rule out a 1-carbon mechanism. Perhaps different cell types within the animal simply have different folate importers that can import some folate analogs and not others?

Somewhat relatedly, the authors find that a folate precursor DHP can induce the egg-laying phenotype. Is there evidence in the literature that nematodes can't convert DHP into folate? It might be easy to investigate using LCMS.

On the same point: The authors conclude that 10F-THF and DHP are signalling molecules to neurons and perhaps signalling for the FOLR-1 receptor. I think that most would argue that 10F-THF and DHP are more different from each other than 10F-THF and the other folates in Sug Fig 3. This issue at least requires some discussion. I think the argument would be that is the dihyropteroate moiety that is engaging in the signalling mechanism and that variants of that would not sufficiently engage the receptor. This is testable if there are other folate analogs that have the dihyropteroate moiety conjugated to other molecules beyond glutamate.

Very few genes in C. elegans are haplo-insufficient. The authors show that heterozygotes of the gon-2 presumptive null suppresses the animal's response to 10F-THF. The authors do not provide any interpretation of this surprising result (the fact that half the dose is insufficient to provide function) and they really should. Furthermore, their concluding sentence of the relevant paragraph states that '…GON-2 is also required for the response to folate/pteroate'. But if it is required, and half the dose is present (likely more than half), why does it have a phenotype. Significant clarification is required here. The 10F-THF insensitivity of the balanced gon-2 allele could be due to something else in the background and with the data presented, cannot be ruled out. If they were to perform the rescue experiments for gon-2 akin to what they did for the folr-1 mutant, I would be more convinced.

Relatedly, without cell/tissue-specific rescue experiments, the authors cannot state that they have shown that GON-2 functions in the same neuronal processes as FOLR-1 (i.e., the first sentence of the third paragraph of the discussion).

The purpose of figure 1c (including its description in the main text) was not entirely clear. It seemed superfluous with a close examination of the folr-1p::FOLR::GFP expression pattern. Furthermore, shouldn't it be localized sub-cellularly at the plasma membrane? It looks ubiquitous in NSM and HSN.

Other Comments
I would have appreciated a bit more information in the introduction about how folate is (mechanistically) used in one-carbon metabolism beyond the one sentence in the first intro paragraph.

The authors use the term 'eggs' instead of 'embryos' throughout, including in figure 1a. Folks in the C. elegans field often use the terms interchangeably, but the authors may want to consider revising to 'embyros' so as not to confuse the non-expert.

The authors characterize pharynx muscle as striated, which it is not.

Good luck getting 'data not shown' past the copy editor- save yourself the trouble and make a sup figure of the transient expression.

The discussion of the details of the truncated ek44 mutant protein is fine but it should be stated that it is likely null because of non-sense mediated RNA decay mechanisms anyway.

Using one molecule with different to infer the details of the diffusion of a second molecule does not make much sense to me because they have different physicochemical properties. Anyway, I don't think its necessary to begin with.

For clarity, the sentence, 'The conversion of DHP to dihydrofolate…' should refer to bacteria. The next sentence should read, "Animals, including nematodes,…' (if indeed that is correct).

Figure 6a- the blues are too similar for my eyes (at least on my computer display).

Do many other species of bacteria make 10F-THF? It is unlikely that elegans' diet (or that of other nematodes) in the wild solely consists of (or even includes) E. coli (or in many niches, even includes E. coli). What would be the conceivable evolutionary consequence of a lack of 10F-THF in the diet?

Reviewer #3:
Remarks to the Author:
The present manuscript demonstrates that folates, here in particular 10-formyl-tetrahydrofolate, activates neural responses in C. elegans, leading to changes in behaviour, here mainly in egg-laying and movements. This is indeed a new and original finding if it can be ultimately proven that the claimed effects are not due to metabolic processing of the tested form of folates and if the hyopothetic mode of action is indeed via a signal to activate serotonergic neurons.

Overall, the manuscript is very well and thoroughly prepared, the methodology is sound (and quite sophisticated) and very much meets the expected standards of the field. The method section provides sufficient details to reproduce the work.

There is, however, a main issue concerning the statement that the study results imply an effect on FOLR-1 independent of metabolism. The authors used DHP as a 10F-THF mimicking compound which cannot be metabolised but shows similar effects as 10F-THF. If this is the case, DHP should produce the same signal as does 10F-THF at FOLR-1, but there is no data provided to support that there is a direct interaction of DHP with FOLR-1. The data provided only indirectly indicates this interaction. I would recommend to elaborate on possible ligand-receptor interactions or ideally to provide data on this possibly based on in-silico methods which can be done quite rapidly and easily.

The second issue is on the use of the "freshly created" 10F-THF. It has to be acknowledged that the authors took the instability of this folate metabolite into consideration and the approach to overcome this problem by converting 5,10-methenyl-THF at a pH of 8.5 sounds intriguing. In the reference (58) provided for this conversion, however, there is only limited information on the effective conversion rate, and any data supporting an efficient conversion would be helpful. As this

is the main compound studied in the manuscript it is crucial to provide data on either the exact concentration of 10F-THF or at least on the gain of 10F-THF produced from 5,10-methenyl-THF. While the authors correctly state that 10F-THF is rather instable, this is also true for THF which has been used in the manuscript as one of the additional folate metabolites studied.

On a minor note, I would suggest to change the title of the manuscript slightly to take into consideration that only the tested form of folates - 10F-THF - had an activating effect while the other folates tested had not.

Response to Reviewers

Our response to the reviewers are indented on both sides.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This study explores the relationship between folate vitamin related molecules, their interaction with the C. elegans FOLR-1 folate transporter, and potential activation of the TRPM-family channel GON-2 in the regulation of egg-laying and dwelling behaviors. The authors first show that FOLR-1 is expressed in two major serotonin-producing and releasing cells, the HSNs and NSMs, which release serotonin to promote egg-laying and dwelling, respectively. The 10F-THF and DHP folate derivatives promote egg laying in briefly starved wild-type animals but not mutant animals lacking the FOLR-1 receptor or the TRPM channel GON-2, both of which the authors show are co-expressed in NSM and HSN. As the induction of egg laying is relatively rapid, within ~10 minutes or so, the authors postulate these folate-related molecules promote egg laying via a signaling, not metabolic, pathway. This conclusion is supported by evidence that 5-methyl-THF, 5-formyl-THF, and THF did not have the same effect. While these metabolic vs. signaling questions are outside the expertise of this reviewer, I do not believe these experiments exclude a metabolic role for these small molecules, for example in biopterin-dependent serotonin biosynthesis that could contribute to the behavior results observed.

> Tetrahydrobiopterin (BH4) is required for serotonin synthesis. However, despite BH4 being a pteridine, and therefore related to folates and pteroates, it is not synthesized from folate or pteroate precursors. Rather, BH4 is synthesized from GTP through a distinct multistep process. There is one pathway that might link BH4 to serotonin levels. BH4 can be oxidized to become dihydrobiopterin (BH2), which does not function in serotonin synthesis. Dihydrofolate reductase (DHFR) can convert BH2 to BH4, thereby making it available for serotonin synthesis. In mammals, folate has been shown to enhance the conversion of BH2 to BH4 by increasing DHFR expression. However, it is not known if this linkage between folate and DHFR levels is conserved in *C. elegans*. Nevertheless, the reviewer's concern that a reduction in folate levels in *folr-1* mutants could reduce serotonin levels has a potential mechanism in other animals. We have addressed the possibility that serotonin levels are reduced in *folr-1* mutants through experiments that are described below.

Because both folr-1 and gon-2 mutants are resistant to 10F-THF and DHP-induced egg laying, and that both genes contribute to germline development, the authors tested their hypothesis that these two membrane proteins interact physically. Using a transient over-expression approach in HEK293 cells, the authors detect co-immunoprecipitation from detergent extractions, suggesting they are part of a complex.

The authors then go on to show that THF and DHP (weakly) can stimulate HSN Ca2+ transient activity. The stimulation of egg laying by 10F-THF similarly depends upon serotonin and HSN function, as it is lost in serotonin-deficient tph-1 mutants or egl-1(dm) animals lacking HSNs. This data is consistent with HSN-released serotonin being required for the 10F-THF effect. The authors then try to determine where FOLR-1 is acting through cell & tissue-specific rescue experiments. Expression of FOLR-1 just in HSN and NSM fails to restore 10F-THF stimulation of egg laying,

suggesting additional sites of action. Co-expression of FOLR-1 in the isthmus and intestinal-valve were required for full rescue which the authors interpret as showing additional roles for FOLR-1 in transport of 10F-THF for its egg-laying behavior effects. This result complicates interpretation of the previous HSN Ca2+ activity results, as the DHP and 10F-THF stimulatory effect and its reduction in folr-1 (and maybe gon-2) mutants may be upstream of HSN.

> We show that expression of FOLR-1 in three tissues in *folr-1* mutants (NSM, the isthmus of the pharynx, and the intestinal valve cells) does not rescue the egg-laying defect (Fig. 5b). However, when FOLR-1 is expressed in HSN plus those tissues, then the egg-laying defect is rescued. This implies that FOLR-1 must be expressed in HSN to promote egg laying. We also show that expression of FOLR-1 in three tissues that does not include NSM (HSN, the isthmus of the pharynx, and the intestinal valve cells) does not rescue, but that expression in those tissues plus NSM rescues, implying that FOLR-1 must be expressed in NSM. Thus, our results suggest that FOLR-1 is required in both NSM and HSN neurons. We describe how a published work (ref. 34) has shown that serotonin released by HSN is taken up by NSM and utilized in NSM-mediated signaling. It is likely that serotonin that is released systemically by NSM can similarly move to the location of the HSN neurons and either be taken up by HSN to enhance its activity and/or contribute to egg laying directly by interacting with the vulval muscles. Previously, it was shown that if HSN is ablated then providing exogenous serotonin can replace HSN function, thus showing that systemically-distributed serotonin can regulate egg laying (ref. 17). Therefore, our results suggest that FOLR-1 functions in both NSM and HSN neurons for the regulation of egg laying. Additionally, as detailed below, we now show that GON-2 is also required in the NSM neurons for this process.

The authors then turn to NSM functions of folr-1 and gon-2 finding a very interesting observation that these mutants have a defect in enhanced-slowing after brief starvation and reencountering of food. The authors describe this in terms of recovery of locomotion after the initial slowing, but the videos suggest the worms may not slow that much in the first place.

> We performed analyses at two different time points relative to the stopping behaviour. An analysis of initial velocity in the first minute is shown in Fig. 6a, and data on longer term recovery of movement was shown in the previous Fig. 6b, c. The initial velocity analysis indicates that *folr-1* mutants have reductions in velocity upon encountering bacteria that are similar to wild type, while *gon-2* mutants maintain elevated velocity longer. We have now added data that shows that by 2 min after encountering bacteria, both *folr-1* and *gon-2* mutants have traveled further distances than wild type, indicating a reduction in the NSM-mediated stopping behaviour. This is shown in a new Fig. 6b. This new data bolsters the previous data that shows that *folr-1* and *gon-2* mutants recover their movement sooner after encountering bacteria (Fig. 6c), indicating that FOLR-1 and GON-2 are required for the full NSM-mediated stopping behaviour.

Overall, there are some interesting data here, and the experiments are well-performed. The issue is the interpretation and how to bring these pieces together in a way that makes sense. Evidence for a direct and functional important interaction where folate activation of FOLR-1 facilitates TRPM channel activity in HSN or other cells remains speculative. The HSNs still show robust Ca2+ activity

in both mutants, although that does not preclude them functioning in some way to modulate cell excitability. The author's results showing FOLR-1 functions in several cells upstream of HSN suggests the Ca2+ effects seen may be indirect. I am concerned about the interpretation by the authors for a signaling vs. metabolic role since most of the data could be explained by changes in serotonin signaling for both egg-laying and slowing behaviors. A prediction would be that these mutants have serotonin deficiency and that exogenous DHP / 10F-THF helps elevate serotonin levels by promoting its synthesis. This could be tested directly through anti-serotonin antibody staining (Tanis et al. 2008) or other, more direct serotonin measurements. Perhaps one of their derivatives cannot contribute to serotonin biosynthesis. Such a result would not be unprecedented, as I think this is why folate and biopterin deficiencies are thought to contribute to depressive behavior. If the authors are convinced this model is unlikely, it would be helpful if they navigate that in the Discussion and/or Results. As it is, it does not seem to be addressed as a possibility even to rule out. Again, this is outside the metabolic expertise of this reviewer, but that may also be true for a generic reader of the paper who might be similarly curious about FOLR-1's role in serotonin biosynthesis.

> The reviewer suggests the possibility that the loss of folate transport in *folr-1* mutants decreases serotonin levels in HSN and NSM, which reduce their functions. As we describe in our previous comment, there is a potential indirect mechanism whereby folate could increase the synthesis of serotonin by increasing DHFR expression (if *C. elegans* is similar to mammals in this regard). To determine if serotonin levels are reduced in *folr-1* and *gon-2* mutants, we have followed the reviewer's suggestion and analyzed serotonin levels using anti-serotonin antibody. We found that in both NSM and HSN neurons, the level of serotonin in *folr-1* mutants was similar to that in wild type. Unexpectedly, we observed that the levels in *gon-2* homozygous and heterozygous mutants were almost two-fold higher level than in wild type. This data is now presented in a new Results section (page 17, lines 359-372) and in a new Supplemental Figure 10. The results indicate that there is no decrease in serotonin levels in NSM or HSN neurons in *folr-1* or *gon-2* mutants. This is consistent with our model that FOLR-1 and GON-2 act upstream of serotonin release to increase calcium signaling in these neurons, as evidenced by increased Ca^{2+} transients in HSN (Fig. 4) and increased Ca^{2+} levels in NSM (Fig 6d, e). While the mechanism by which GON-2 activity promotes increased Ca^{2+} transients remains unclear, we now cite a study showing that a TRPML Ca^{2+} channel similarly elevates Ca^{2+} transients in *Drosophila* astrocytes (ref. 41).

Major points:
- Is there any prior evidence from other studies or systems that FOLR-1 and GON-2 would interact? If so, the authors should include such a connection because it is a bit of a leap. Why would such an interaction be expected and how would it facilitate either proteins biological functions? If the authors are really convinced the functions of these two proteins is integral, it may be worth speculating on its functional significance.

> While our manuscript was under review, a paper was published in *Nature Communications* that showed that in *Xenopus* neural plate cells, folate addition increases the number of Ca^{2+} transients, and the number of Ca^{2+} transients is reduced by knockdown of FOLR1 (Balashova et al., 2024, vol. 15: 1642). Their study also showed that FOLR1 is required for neural tube closure, and that neural tube

defects due to partial FOLR1 knockdown could be rescued by the addition of a pteroate. These results suggest that the role of FOLR1 in mediating calcium entry through a non-metabolic pathway is evolutionarily conserved. Our manuscript is complementary to their paper, and includes several advances that are not covered in their study.

1) Regulation of neuronal activity. We show that FOLR-1 regulates neuronal activation, while their study focuses on neural plate cells forming the neural tube.

2) The folate induction of Ca^{2+} transients requires FOLR-1. We show that mutation of FOLR-1 blocks the increase in Ca^{2+} transients in response to folate. In contrast, their paper only shows that FOLR1 knockdown reduces the number of spontaneous Ca^{2+} transients; it does not show that FOLR-1 knockdown abrogates the increase in Ca^{2+} transients in response to folate.

3) The increase in Ca^{2+} transients does not involve one-carbon metabolism. We show that the increase in Ca^{2+} transients occurs in response to pteroate, implying a non-metabolic pathway, whereas their research did not investigate the effect of pteroate on Ca^{2+} transients (only on neural tube closure).

4) TRPM Calcium channel GON-2. We identify the TRPM calcium channel GON-2 as a contributor to these folate- and FOLR-1-regulated processes, while their study did not identify proteins involved in calcium entry.

5) Physiological Folate levels. Our study uses folates at more physiological concentrations (nM) compared to their study, which uses supraphysiological µM levels of folates (up to 300 µM). The level of folates in human serum are in the low nM range, and thus our study demonstrates that folates can impact neural activity at more physiological levels.

Together with their study, our findings provide a significant advancement to the field. We now include a discussion of their results and its evolutionary implications in the Discussion section (page 20-21, lines 439-446). The interaction of FOLR-1 with a Ca^{2+} channel makes sense in light of the observation that folate regulates Ca^{2+} entry in multiple organisms through an FOLR-1-dependent process. We propose the possibility that FOLR-1 physically interacts with GON-2 to modulate the activity of GON-2 to promote calcium entry in response to folate (page 19, lines 399-402).

- The authors conclude from their co-immunoprecipitation experiments that FOLR-1 and GON-2 “interacted strongly with each other,” but there is no quantitative evidence to support such a determination. The authors do not report the extent to which these co-expressed proteins are associated with each other. The interaction may also be indirect. The extent could be estimated from densitometric quantitation relative to how much protein was present in the starting lysate. The authors should report. either in the Materials & Methods or the Figure 3C legend itself, how much lysate was run and whether the exposures presented in different gel cutouts are comparable.

To characterize the extent of interaction, we have determined the percentage of total protein that co-precipitated in the immunoprecipitations. This is now shown in a graph as Fig. 3d. We found that on average 15–16% of FOLR-1-HA and GON-2-FLAG co-precipitated with the other protein, significantly higher than for the control proteins.

Regardless, the authors should temper their conclusions about the relative strength of the interaction absent additional evidence. The proteins could just be sticky. If demonstrating such a direct interaction was critical to the authors, they could perform additional control experiments like performing the co-immunoprecipitation experiment from a mixed detergent lysate prepared from separate transgenic cells expressing only a single protein.

> We have removed the description that the proteins interact strongly (page 11, lines 215-217).
>
> We tested whether mixing lysates from cells that independently expressed FOLR-1 or GON-2 would allow interaction. In these experiments, interaction did not rise above background levels. However, it is not surprising that the two proteins fail to interact because FOLR-1 and GON-2 are membrane-localized proteins that are only added together after solubilization with detergent. There are many examples from signal transduction that show that physical interactions only occur when two proteins are membrane localized.

- In Figure 4a and 4b, it's unexpected that the egl-1(dm) mutant lays a similar number of eggs as the tph-1 mutant, considering they are much more Egl. I would expect them to be less likely to lay eggs under these conditions, certainly more defective than wild-type animals even though the number of eggs laid are not dramatically different. Admittedly, the M9-agarose assay used here is atypical and may lead to non-intuitive results. It seems (and the authors state this directly) that this assay does have some variability across experiments, so it would be useful to know if wild-type animals were assayed alongside the tph-1 and egl-1 mutant data shown here. If that data was collected at the same time, it should be shown.

> There is variability in the overall level of egg laying between experiments. Therefore, as suggested, we have repeated the egg laying experiment with wild type, *tph-1*, and *egl-1* mutants assayed at the same time. This analysis shows that both *tph-1* and *egl-1* mutants lay significantly less eggs than wild type and are not responsive to the addition of 10F-THF (see new Fig. 5a). While *egl-1* has the name *egg laying defective*, *tph-1* mutants are also egg laying defective with a reduced rate of egg laying and the accumulation of eggs in the uterus (ref. 29). We now cite this paper and alert the reader to this fact (page 12, lines 249-252).

- It is surprising that the gon-2/hT2 mutant is effective as a heterozygote. It would be helpful if the authors could speculate in the Discussion why you feel this mutant has as strong phenotype as a het. They may not be able to get very far, although it is not unprecedented for signaling mutants that affect egg-laying behavior to have semi-dominant effects (although this reviewer is having difficulty finding published papers that report that fact directly).

> We now cite four egg-laying defective mutants that affect egg-laying rates as heterozygous loss-of-function mutations, thereby providing precedent for the *gon-2* heterozygous effect on egg laying in response to folate. However, we point out that heterozygous reduction of *gon-2* also affects the NSM-mediate stoppage, and so the hemizygous effect is not limited to egg laying. One possibility that we discuss is that interaction between FOLR-1 and GON-2 requires the normal level of GON-2 expression. This is discussed on pages 19-20, lines 412-421.

- How were animals synchronized? The methods describe animals as having a single row of eggs, but that would likely vary among the genotypes used in this study (for example, egl-1(dm) mutants). Did the authors use other methods, for example staging from timed egg lays, bleached egg preps, or L4's? Please add that information.

> We selected adult hermaphrodites from cultures where the leading edge of the progeny were one-day old adults. These plates were obtained by daily cloning of 8 to 12 L4-stage larvae onto large, 10 cm plates seeded with OP50 bacteria. Three days later, the leading edge of the progeny were one-day old adults, and hermaphrodites with one-row of eggs were selected from these plates for the experiments. This information is now included in the Methods section, page 23, lines 487-491.

- The data in 6b and 6c do not appear to be normally distributed. It may make more sense to use non-parametric statistical tests and focus on median vs. means.

> We thank the reviewer for pointing out the non-normal distribution. We now use the non-parametric Kruskal-Wallis test followed by multiple comparisons using Dunn's test. The experiment on recovery times for the NSM-mediated stopping behaviour was performed both with and without fluoxetine (which blocks serotonin reuptake to increase the stopping phenotype). Using the non-parametric tests, the *folr-1* mutant recovery time without fluoxetine is not significantly shorter than wild type. The gon-2 mutant recovery times remain statistically shorter, as do the recovery time for *folr-1* mutants with fluoxetine. We have moved the previous Fig. 6b graph (showing recovery time without fluoxetine) to Supplementary Fig. 9b. It has been replaced with the analysis of distance traveled after encountering bacteria, which shows that *folr-1* mutants move further than wild type (discussed above).

Reviewer #2 (Remarks to the Author):

Review of Peesapati et al., "Folate activates serotonergic neurons to control C. elegans behaviour'.

The authors present an interesting story about how a specific folate that is food (bacteria)-derived impacts neuronal calcium activity and in turn, the behaviour of the nematode C. elegans. As far as the authors report (I am not an expert in folate biology), this appears to be the first evidence beyond expression patterns in mice of a folate receptor being involved in neuronal signalling, which is interesting. The authors provide substantial genetic evidence that the folate receptor (FOLR-1) functions in select neurons and tissues that may mediate folate transport. They also provide direct and circumstantial lines of evidence for FOLR-1 interaction with the GON-2 calcium channel. A mechanistic model is suggested (folate interaction with FOLR-1 modifies GON-2 channel activity) in the discussion, but not tested in a heterologous assay such as in Xenopus oocytes.

Comments

Eighth paragraph of the results (this would have been easier with line numbers in the manuscript): I am not convinced that one-carbon metabolism is not relevant to the phenotypes being studied

based on this paragraph alone (which is what they conclude at the end of the paragraph). The authors report that only 10F-THF, but not other physiological folates (what is a physiological folate btw?), can modulate germ cell proliferation. They then go on to report that several folates, including 10F-THF can rescue elegans folate deficiency. Is a germ cell proliferation phenotype not part of folate deficiency? I find this confusing. It seems that some phenotypes are sensitive only to 10F-THF while others are not. It is not clear to me how this can rule out a 1-carbon mechanism. Perhaps different cell types within the animal simply have different folate importers that can import some folate analogs and not others?

> We have now clarified this information. We have previously shown that *C. elegans* that are starved for folate (and have very few germ cells) can have their folate deficiency rescued by the addition of different folates that are involved in one-carbon metabolism (previously referred to as physiological folates). Some of these folates can rescue the folate deficiency better than 10F-THF. Notably, FOLR-1 is not required for the basal rate of germ cell proliferation, as *folr-1* mutants have normal numbers of germ cells. However, we found that the addition of 10F-THF can increase germ cell proliferation above basal levels. This mechanism requires FOLR-1 and is specific for 10F-THF but not other cellular folates that we tested, even though some of those folates could rescue folate deficiency better than 10F-THF. Thus, 10F-THF acts specifically to increase proliferation above the normal basal level, and FOLR-1 is required for this increase above basal levels. To explain this adequately, we have moved this discussion from the Results section to the Introduction (page 4, lines 56-63). We no longer list this as evidence for a non-metabolic role for particular folates, rather we merely suggest that it shows a decoupling of the ability of folates to function in one-carbon metabolism from their ability to increase germ cell proliferation above the basal proliferation rate.
>
> As suggested, we have added line numbers to make it easier to follow the changes.

Somewhat relatedly, the authors find that a folate precursor DHP can induce the egg-laying phenotype. Is there evidence in the literature that nematodes can't convert DHP into folate? It might be easy to investigate using LCMS.

> We now explain in an expanded paragraph in the introduction that pteroates (including DHP) cannot be converted to folates in animals because all animals lack dihydrofolate synthase, which is required for the conversion of pteroates to folates in bacteria, fungi, and plants. We now provide a more definitive reference that indicates that pteroates cannot be utilized in one-carbon metabolism in animals (ref. 9). That paper also describes how the bacteria *Lactobacillus casei* (which is used for the biological assay for folate levels) lacks functional dihydrofolate synthase. *L. casei* can take up and utilize any type of folate for one-carbon metabolism, but cannot utilize pteroates for one-carbon metabolism, as they cannot convert pteroates to folates. The inability of *L. casei* to utilize pteroates demonstrates that even bacteria cannot utilize pteroates for one-carbon metabolism (without first converting them to folates). We also cite our work showing that *C. elegans* that are starved for folate cannot be rescued by providing pteroates, but can be rescued by providing different types of individual folates (ref.

8). This implies that *C. elegans*, like all animals, cannot use pteroates for one-carbon metabolism. This information is now on pages 4-5, lines 64-83.

On the same point: The authors conclude that 10F-THF and DHP are signalling molecules to neurons and perhaps signalling for the FOLR-1 receptor. I think that most would argue that 10F-THF and DHP are more different from each other than 10F-THF and the other folates in Sug Fig 3. This issue at least requires some discussion. I think the argument would be that is the dihyropteroate moiety that is engaging in the signalling mechanism and that variants of that would not sufficiently engage the receptor. This is testable if there are other folate analogs that have the dihyropteroate moiety conjugated to other molecules beyond glutamate.

We do not know the reason that 10F-THF and DHP are able to activate the FOLR-1-dependent process while other folates are unable to do so. A conclusive analysis of this difference is beyond the scope of this manuscript. We now indicate that there are potential evolutionary reasons that *C. elegans* evolved to respond to 10F-THF and DHP but not to other folates. Both 10F-THF and DHP are highly unstable relative to other folates, and thus provide a signal with a shorter half-life that can link the signal to the ingestion of bacteria more directly. The use of an unstable signal also has the potential to allow *C. elegans* to distinguish between high-quality and low-quality food sources, e.g., live vs. dead bacteria, with the latter presumably lacking the unstable 10F-THF and DHP. A discussion of this issue is now presented on page 20, lines 422-438.

Very few genes in C. elegans are haplo-insufficient. The authors show that heterozygotes of the gon-2 presumptive null suppresses the animal's response to 10F-THF. The authors do not provide any interpretation of this surprising result (the fact that half the dose is insufficient to provide function) and they really should. Furthermore, their concluding sentence of the relevant paragraph states that '…GON-2 is also required for the response to folate/pteroate'. But if it is required, and half the dose is present (likely more than half), why does it have a phenotype. Significant clarification is required here. The 10F-THF insensitivity of the balanced gon-2 allele could be due to something else in the background and with the data presented, cannot be ruled out. If they were to perform the rescue experiments for gon-2 akin to what they did for the folr-1 mutant, I would be more convinced.

Relatedly, without cell/tissue-specific rescue experiments, the authors cannot state that they have shown that GON-2 functions in the same neuronal processes as FOLR-1 (i.e., the first sentence of the third paragraph of the discussion).

We have now performed the experiment requested by the reviewer. We expressed GON-2 in specific tissues of *gon-2* heterozygotes to rescue the inability to respond to 10F-THF to increase the rate of egg laying. *gon-2* heterozygotes are used because *gon-2* homozygotes are sterile. We found that expression of GON-2 in NSM alone, or NSM + HSN, or NSM + HSN + the isthmus of the pharynx + the pharyngeal-intestinal cells can all rescue the egg laying defect. In contrast, expression of GON-2 in HSN alone does not rescue the egg-laying defect. This indicates that GON-2 is required at least in NSM neurons to allow animals to respond to folate to increase egg laying. This data is shown in Supplementary Fig. 8. Because of the heterozygous background, we cannot determine if GON-2 is also

required in HSN neurons (as they already have half the normal level of GON-2). However, this result reinforces our previous finding that FOLR-1 must be expressed in NSM to promote egg laying. As we have described, our model is that serotonin released by NSM is captured and utilized by HSN and/or it directly stimulates the vulval muscles to promote egg laying. One of the major conclusions of the manuscript is that FOLR-1 and GON-2 function in neurons to promote their activity in response to folates. This major point is made by demonstrating that GON-2 functions in NSM neurons. It is not necessary to also show that GON-2 functions in HSN neurons to make this point.

We do not know why hemizygous levels of GON-2 are sufficient to negate the effect of folate on egg laying or the NSM-mediated stopping. For the NSM stopping behaviour, we show that the *gon-2* heterozygous phenotype is similar to that of the *gon-2* homozygous phenotype. As described above, we speculate that the *gon-2* hemizygous effect could result from GON-2 and FOLR-1 only functionally interacting when there are sufficient levels of GON-2 (pages 19-20, lines 412-421).

The purpose of figure 1c (including its description in the main text) was not entirely clear. It seemed superfluous with a close examination of the folr-1p::FOLR::GFP expression pattern. Furthermore, shouldn't it be localized sub-cellularly at the plasma membrane? It looks ubiquitous in NSM and HSN.

We have reworded the text to clarify the purpose of Fig. 1c (pages 6-7, lines 121-127). We believe that Fig. 1c is useful in allowing readers to more readily assess the intracellular localization of FOLR-1::wrmScarlet without the signal in neurites being obscured by the expression in the isthmus of the pharynx. A long exposure was used for in Fig. 1c to allow visualization of the thin neurites. However, a long exposure saturates the signal in the cell body. We now provide Supplementary Fig. 4, which shows confocal microscope images of FOLR-1::wrmScarlet localized to regions of the plasma membrane (which is marked by myristoylated mNeonGreen).

Other Comments
I would have appreciated a bit more information in the introduction about how folate is (mechanistically) used in one-carbon metabolism beyond the one sentence in the first intro paragraph.

We now include a more in depth description of the role of one-carbon metabolism in the cell (page 3, lines 32-40).

The authors use the term 'eggs' instead of 'embryos' throughout, including in figure 1a. Folks in the C. elegans field often use the terms interchangeably, but the authors may want to consider revising to 'embyros' so as not to confuse the non-expert.

The use of 'eggs' rather than embryos is to denote that the embryos are surrounded by an eggshell and are laid outside the animal. We now clarify this terminology for the reader on page 5, lines 90-91.

The authors characterize pharynx muscle as striated, which it is not.

We appreciate this correction, and have updated the wording (page 5, line 98-99).

Good luck getting 'data not shown' past the copy editor- save yourself the trouble and make a sup figure of the transient expression.

We have added additional images of FOLR-1::GFP expression as Supplementary Fig. 3, and removed 'data not shown'.

The discussion of the details of the truncated ek44 mutant protein is fine but it should be stated that it is likely null because of non-sense mediated RNA decay mechanisms anyway.

We now provide qRT-PCR results that show that the expression of *folr-1* mRNA in *folr-1(ek44)* is 1/15th the level in wild-type. As suggested, we now state that nonsense mediated decay is likely responsible for this reduced level of expression (pages 7-8, lines 144-147).

Using one molecule with different to infer the details of the diffusion of a second molecule does not make much sense to me because they have different physicochemical properties. Anyway, I don't think its necessary to begin with.

We agree that is not an ideal solution, but nevertheless think that it provides a useful approximation of the concentration of folate/pteroate on the top of the agar plate. We now add an extra note of caution when interpreting the results (page 8, lines 153-157).

For clarity, the sentence, 'The conversion of DHP to dihydrofolate…' should refer to bacteria. The next sentence should read, "Animals, including nematodes,…' (if indeed that is correct).

We have added the suggested information (page 4, lines 68-72).

Figure 6a- the blues are too similar for my eyes (at least on my computer display).

We have changed the colors on Fig. 6a to make them more distinct (and now also colorblind friendly), and used the same color scheme for the genotypes in Fig. 6b, c and Supplementary Figs 9b, 10c, d.

Do many other species of bacteria make 10F-THF? It is unlikely that elegans' diet (or that of other nematodes) in the wild solely consists of (or even includes) E. coli (or in many niches, even includes E. coli). What would be the conceivable evolutionary consequence of a lack of 10F-THF in the diet?

10F-THF is one of the seven folates involved in the one-carbon metabolism cycle. 10F-THF is the specific folate that donates one-carbon unit to create two purine precursors. Current data suggests that all species in the seven kingdoms of life utilize the one-carbon metabolism cycle, and therefore, 10F-THF is present in all these species. As described above, we propose that the reason 10F-THF is used as a marker of the presence of bacteria is because 10F-THF is the most unstable

cellular folate. The short half-life of 10F-THF would allow it to be used to signal the immediate availability of bacteria, and potentially distinguish between high- and low-quality food. Our discussion of why 10F-THF and DHP may be evolutionarily selected as markers of bacteria is presented on page 20, lines 422-438.

Reviewer #3 (Remarks to the Author):

The present manuscript demonstrates that folates, here in particular 10-formyl-tetrahydrofolate, activates neural responses in C. elegans, leading to changes in behaviour, here mainly in egg-laying and movements. This is indeed a new and original finding if it can be ultimately proven that the claimed effects are not due to metabolic processing of the tested form of folates and if the hyopothetic mode of action is indeed via a signal to activate serotonergic neurons.

Overall, the manuscript is very well and thoroughly prepared, the methodology is sound (and quite sophisticated) and very much meets the expected standards of the field. The method section provides sufficient details to reproduce the work.

There is, however, a main issue concerning the statement that the study results imply an effect on FOLR-1 independent of metabolism. The authors used DHP as a 10F-THF mimicking compound which cannot be metabolised but shows similar effects as 10F-THF. If this is the case, DHP should produce the same signal as does 10F-THF at FOLR-1, but there is no data provided to support that there is a direct interaction of DHP with FOLR-1. The data provided only indirectly indicates this interaction. I would recommend to elaborate on possible ligand-receptor interactions or ideally to provide data on this possibly based on in-silico methods which can be done quite rapidly and easily.

Our attempts to predict the structure of the *C. elegans* FOLR-1 protein using in-silico methods have not produced satisfactory results. We have been careful to word the manuscript to denote that our data indicates that 10F-THF and DHP are able to induce the activation of NSM and HSN neurons to regulate the behaviours that they control, and that this activation requires the folate receptor FOLR-1. Understanding the biochemical interactions involved is a worthwhile goal that we plan to pursue in the future, but it is beyond the scope of the current manuscript.

The second issue is on the use of the "freshly created" 10F-THF. It has to be acknowledged that the authors took the instability of this folate metabolite into consideration and the approach to overcome this problem by converting 5,10-methenyl-THF at a pH of 8.5 sounds intriguing. In the reference (58) provided for this conversion, however, there is only limited information on the effective conversion rate, and any data supporting an efficient conversion would be helpful. As this is the main compound studied in the manuscript it is crucial to provide data on either the exact concentration of 10F-THF or at least on the gain of 10F-THF produced from 5,10-methenyl-THF.

We now provide absorbance data showing that the bulk of 5,10-methenyl-THF is converted to 10F-THF within 15 minutes (Supplementary Fig. 11).

While the authors correctly state that 10F-THF is rather instable, this is also true for THF which has been used in the manuscript as one of the additional folate metabolites studied.

We are aware that reduced folates are unstable. Therefore, we store them under nitrogen gas in liquid nitrogen, dissolved in 2% sodium ascorbate (to reduce oxidation). However, 10F-THF is more unstable than THF. Information on the instability of 10F-THF comes from our co-author Jacob Selhub, who is an expert in folate biochemistry, and from the documentation at Schircks Laboratories. Schircks Laboratories is the foremost commercial synthesizer of folate- and pteroate-related compounds. They indicate on their website that they do not sell 10F-THF because it is too unstable, while they do sell THF.

On a minor note, I would suggest to change the title of the manuscript slightly to take into consideration that only the tested form of folates - 10F-THF - had an activating effect while the other folates tested had not.

As suggested, we have changed the title to "A specific folate activates serotonergic neurons to control *C. elegans* behaviour" to reflect that other cellular folates are not able to activate the pathway (with the exception of 5,10-methenyl-THF, which is converted to 10F-THF at physiological pH).

Other Changes:

• the previous Supplementary Fig. 3 (on folate structures) is now Fig. 1 because it is mentioned earlier in the text. The numbering of the other previous Supplementary Figures have been shifted because of this change and the inclusion of new Supplementary Figures.

Reviewer #1

(Remarks to the Author)
The authors have addressed all my major concerns, and I support publication of the manuscript.

Some remaining minor comments:

- My suggestion about mixing lysates in the co-immunoprecipitation experiment was meant to test whether the interaction artefactually occurred upon detergent lysis. Most authentic membrane protein-protein interactions would fail to associate in such mixed extracts; they are only observable and retained when first present in the starting membranes. That you failed to observe such an interaction above background is evidence to support such a direct, specific interaction.

- On your list of semidominant Egl's, I would stick to egl-10 and egl-30 as examples. egl-7 and egl-40 mutants have not been cloned (or at least, their gene identity not been published outside of worm meeting abstracts). They are also described as dominant, possibly gain-of-function alleles, and may not be the best examples of haploinsufficiency.

(Remarks to the Editor)

Reviewer #2

(Remarks to the Author)
I have reviewed the authors response to my comments and appreciate the amount of effort (writing and experimental) that they put into addressing my concerns. I have no further concerns with the manuscript.

(Remarks to the Editor)

Reviewer #3

(Remarks to the Author)
The authors made enormous efforts to address the issues raised by the reviewers. From my point of view, all of these have now been clarified partly by generating additional data and experiments, and the submission has even improved over the already great initial version of the manuscript. There remain no further issues which would keep the manuscript from being published.

(Remarks to the Editor)

Response to Reviewers

Our response is indented on both sides.

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

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Some remaining minor comments:

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We agree with the assessment of the reviewer.

- On your list of semidominant Egl's, I would stick to egl-10 and egl-30 as examples. egl-7 and egl-40 mutants have not been cloned (or at least, their gene identity not been published outside of worm meeting abstracts). They are also described as dominant, possibly gain-of-function alleles, and may not be the best examples of haploinsufficiency.

We have removed the references to *egl-7* and *egl-40* mutants (and retained our description of *egl-10* and *egl-30*), as suggested. We have kept the same three reference citations, as references 48 and 49 describe *egl-10* and *egl-30*, while reference 30 provides a broader overview of more potential egl genes that may reduce egg laying rates as heterozygous loss-of-function alleles (reference 30 is also cited elsewhere in the manuscript). The modified sentence is on lines 416-417. We thank the reviewer for their suggestions to improve the manuscript.

Reviewer #2 (Remarks to the Author):

I have reviewed the authors response to my comments and appreciate the amount of effort (writing and experimental) that they put into addressing my concerns. I have no further concerns with the manuscript.

We thank the reviewer for their help improving the manuscript.

Reviewer #3 (Remarks to the Author):

The authors made enormous efforts to address the issues raised by the reviewers. From my point of view, all of these have now been clarified partly by generating additional data and experiments, and the submission has even improved over the already great initial version of the manuscript. There remain no further issues which would keep the manuscript from being published.

We thank the reviewer for their help improving the manuscript.

Other changes not covered in the Author Checklist:
We have slightly shortened the Abstract to bring it to the 150 word limit.

We have removed two references (the previous references 40 and 56) to bring the total number of references to the limit of 70.