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Monoamines and neuropeptides interact to inhibit aversive behavior in *Caenorhabditis elegans*

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1st Editorial Decision 22 June 2011

Thank you for submitting your manuscript to the EMBO Journal. Your manuscript has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting, but also indicate that that some more analysis is needed to fully support the conclusions and for the manuscript to have the full impact of an EMBO Journal paper. I will not repeat all the issues here as they are clearly indicated below. Given the referees' comments I would like to invite you to submit a suitably revised manuscript should you able be to address the concerns raised in full. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the concerns raised at this stage.

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Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE REPORTS

Referee #1

In this paper, Wragg et al identify two octopamine receptors that modulate avoidance behaviors in C. elegans. The authors previously showed that the OCTR-1 receptor inhibited the activity ASH-dependent responses to dilute octanol in the presence of 4 mM octopamine. Here they provide evidence that a second octopamine receptor, SER-3, acts in ASH to antagonize this inhibition in the presence of 10 mM octopamine. A third octopamine receptor, SER-6, appears to function in other neurons to inhibit avoidance of concentrated octanol. This SER-6 effect requires neuropeptides that are likely to be released from SER-6-expressing neurons.

Overall, this is a very interesting paper that makes important points about how modulation of behavioral states occurs in C. elegans. One one hand, the demonstration that two octopamine receptors appear to function in the same neuron but in opposite ways highlights the intracellular complexity of neuromodulatory response pathways in neurons. The results with SER-6 and the neuropeptides identifies another level of complexity in which different interact, possibly as neurohormones, to control each other's release and together alter the properties of neural circuits. I think this work is certainly of sufficient interest and novelty for EMBO.

My major criticism is that some of the experiments identifying cellular sites of action are missing important controls. Since the paper relies on using behavior to make inferences about the activities of neurons in a complex circuit it is critical that the cells where the different receptors act are identified as conclusively as possible. In cases where cell-specific promoters are not available, all rescue and RNAi phenocopy experiments need to be done with at least two promoters that overlap only in the cell of interest. Also, negative controls showing lack of rescue with a promoter expressed outside the cell of interest are needed, especially for RNAi experiments where concerns about RNAi spreading are still present in the field.

Major Comments:

1. Regarding SER-3 acting in ASH: Only sra-6::RNAi experiments are shown. Since this promoter also expresses in ASI and PVQ, a second promoter expressing in ASH but not ASI and PVQ should also be used to show that ASH is the relevant cell for RNAi phenocopy. An off-target control using a promoter not expressed in ASH should also be provided as a negative control for RNAi spreading. Finally, to test sufficiency as well as necessity of ASH expression for SER-3 modulation, cell-specific rescues of the ser-3 mutant should be performed in ASH, with an off-target control again provided.

2. To understand the role of SER-6 in ASH modulation, more work controls need to be provided to establish a cellular site of action. Since all transgenic lines in the paper rescue the mutant and all RNAi lines affect the behavior, it is difficult to say conclusively that these are the critical cells. At a minimum it is important to provide negative controls (for example using promoters whose expression does not overlap with the cells giving rescue/RNAi phenocopy) to show specificity for these effects.

3. In figure 3, the authors use eat-4RNAi to infer that glutamate is not required for the ASI/AWB effects. Have
they done this experiment with unc-13RNAi, as they did for the ADL neurons? This would seem a more definitive way to test the
requirement for synaptic vesicle transmission.

4. In determining the site of action for npr-15, the authors conclude that npr-15 must act in AWC based on the nlp-1::npr-15RNAi phenotype, though nlp-1 is expressed in at least 10 different classes of neurons. The authors assume that npr-15 can only act in the cells where they observed expression of their GFP, but this puts too much faith in reporter transgene, even a rescuing one. To conclude that AWC is the site of action, they need to use a least one other independent promoter that expresses in AWC but not the other cells in the nlp-1 expression domain. As with other rescue experiments, they also need to include at least one off-target RNAi transgene as a control.

5. The authors also state that ASE has been shown to be involved in attraction but not avoidance. However, activation of ASER (the neuron expressing npr-18) has been shown directly to evoke avoidance behavior, not suppress it (Suzuki, Nature 2008). Thus, enhancement of ASER activity would be predicted to enhance avoidance rather than attraction. Note that ASE has been implicated in avoidance behavior to CO2 (Bretscher, Neuron 2011).

6. There is quite a bit of data (for example, nlp-7;ser-6 and nlp-9,ser-6 double mutants) that is mentioned but not shown. Much of this includes controls that are important for the paper. These results should be provided, at the very least as supplemental figures.

7. Essentially all the experiments in this paper look at effects of exogenous octopamine. Are any of the effects of receptor mutants mimicked by octopamine-deficient mutants? It might be useful to have a little more discussion about the potential biological significance of the modulatory pathways described here.

Minor points:

1. Nomenclature: In all figures, overexpression lines should be labeled consistently (instead of using different labels like ser-6::ser-6, ser-6+, XS). Explain in figure legends what '+' and 'XS' means.

Also, for transgenic rescue experiments, the actual promoter should be stated in the figure legend, rather than using constructions such as "ASI::npr-15RNAi". This is particularly true for cases like "ASH::ser-3RNAi" which are not precisely cell-specific. (It is also not accurate to refer to the sro-1::ser-6 transgene as "ADL::ser-6" since it is also expressed in the SIA neuron.)

Finally, although nomenclature for gene fusions is not completely consistent in the literature, I think the use of constructions like pser-3::ser-3::gfp is confusing. Since four-letter gene names are now in use, pser-3 looks like it refers to a different gene from ser-3. According to http://wiki.wormbase.org/index.php/UserGuide:Nomenclature, the recommended usage for gene all gene fusions is, for example, ser-3::GFP , with an explanation in the legend if necessary to distinguish transcriptional from translational fusions.

2. Discussion of the literature: There has been a lot of work on avoidance behavior in C. elegans, and the authors' discussion of this background seemed somewhat narrow. For example, there have been at least three studies published recently by other groups describing monoamine and/or neuropeptide modulation of ASH in response to food (Ezak, PlosOne 2010; Ezcurra, EMBO 2011; Singh, Curr Biol 2011). It is strange that none of these are cited, especially since they support the authors' point that ASH modulation is complex.

3. For clarity, please specify in text or figure legend which cells are expressing GFP in fig 3D.

Referee #2

This paper outlines a thorough dissection of the complex circuit controlling octopaminergic inhibition of the worm's response to 1-octanol. Expanding on earlier work, they show that SER-3 antagonizes OCTR-1 in ASH, thereby preventing OA inhibition of aversion responses to dilute octanol. OA inhibition of responses to 100% octanol were not dependent on either octr-1 or ser-3, but ser-6, which the authors identified as an octopamine receptor by heterologous expression in Xenopus oocytes. Cell-specific rescue and overexpression experiments suggest that SER-6 functions in ADL, AWB, and ASI to inhibit aversive responses to 100% octanol. Testing strains with disrupted neuropeptide expression, the authors identified several peptides encoding genes required for OA inhibition of responses to 100% octanol. Based on their expression pattern, as well as cell-specific RNAi, the authors link these peptides to ADL, AWB, and ASI. The authors go on to implicate neuropeptide receptors required for OA inhibition of responses to 100% octanol and show evidence that the receptors are function in chemosensory neurons ASE and AWC. This is a well-written paper; the authors did an extremely thorough evaluation and there are only a few suggestion to strengthen an already strong story.

Major Issues

What is the AWC-specific promoter for eat-4 knockdown (Fig 7D)? It would be more convincing if this promoter was used to rescue or knockdown npr-15 and thereby confirm it functions in AWC. Similarly, why not use the gcy-5 promoter to knockdown npr-18 expression specifically in ASER?

Seeing as your measure is 'time to reverse', it is essential to know how your genetic manipulations affect spontaneous reversal rate. Without this data, it is unclear if your effects were caused by changes in octanol sensitivity or just changes in basal locomotion. For each strain, it would be informative to have a negative control (ie 0% octanol), so readers have an idea of the spontaneous reversal rate of your animals.

One assumption throughout the paper is that the response to 100% octanol is ASH-mediated, ie "SER-6 functions in the ADL, AWB and ASI sensory neurons to inhibit ASH-mediated aversive responses." Chao et al. (2004) found that worms without ASHs responded "reasonably well" to 100% octanol off of food. Therefore your responses of interest may not actually be driven by ASH. This needs to be addressed early on in the paper. In figure 3E, it would be informative to see ASH eat-4 and egl-3 knockdown strains. Knockdown of eat-4 would presumably mostly silence the ASH component of the worm's response to 100% octanol.

Minor comments

Page 8, line 1: The text cites: "Figures 1E-G", but there is no figure 1G.

Page 9, line 8: The text states that "OCTR-1 does not appear to be involved in the OA inhibition of aversive responses to 100% 1-octanol," but figure 2a shows that octr-1 mutants respond more quickly than wild-type in the presence of 10mM OA. Please explain.

Page 11, line 14: The text states: "Conversely, the expression of ser-6 in the ADLs, AWBs or ASIs of wild type animals inhibited basal aversive responses off food." From the graph and the statistical comparison (ie ** = different from wild-type in the presence of OA), it seems that expression of ser-6 in ADL and AWB did NOT inhibit basal aversive responses.

Page 29, line 8: "Similarly, in C. elegans, OA has no effect on basal aversive responses to submaximal ASH stimulation" Doesn't Figure 1a show that it does? N2 tested in the presence of OA and 5-HT respond slower than worms tested n the presence of 5-HT alone.

How does ser-6, eat-4, and egl-3 knockdown (Fig 3E) affect 100% octanol responses in the absence of OA?

Referee #3

In this manuscript, Wragg et al. provide strong evidence for the role of peptidergic signaling in the regulation of octopamine mediated reversal response to octanol. This was performed using mostly genetic and behavioral techniques in the model organism C. elegans, and these results have implications in signaling pathways that mediate anti-nociception to noxious stimuli. The authors identified two octopamine (OA) receptors that negatively regulate reversal behavior to octanol: ser-3, which functions in ASH neurons and are activated by high OA concentration, and ser-6, which functions in ASI (and possibly in AWB and ADL) neurons and are activated by OA only in response to high octanol purity. Furthermore, the authors perform a systematic analysis of neuropeptide signaling to identify specific neuropeptides released from ASI, ADL and AWB neurons and screen for receptors on ASE and AWC neurons that mediate the inhibition of reversal by OA.
This work provides insights into the identities of the neuropeptides and the neuropeptide receptors that mediate in octopamine-mediated reversal behavior to octanol in C. elegans and the cells in which they might act.
The authors deduced the neuronal types in which some of these genes may act by GFP reporters and by RNAi knockdown using cell-specific promoters. The selection of some promoters for tissue specific RNAi was not clear and is noted below. The authors appeared to use non-outcrossed versions of mutants obtained from knockout consortia. This is generally a problem because

background mutations may affect phenotypes of interest. In addition, the authors report that overexpression of some neuropeptides and receptors caused overexpression phenotypes making the claims of "rescue" unfounded. However, the authors addressed this issue by confirming most mutant phenotypes they reported by RNAi.
The main concern with this paper is that the results may be of limited interest for publication in EMBO for two reasons: First, the idea that neuropeptide signaling regulates aspects of sensory behavior has been well established by the previous work of the authors and several other groups using the C. elegans model. Several studies have gone on to directly show sensory invoked calcium changes in specific neurons, neuropeptide-receptor interactions, etc. Second, although the authors propose that specific neuropeptides are secreted from specific cells in response to sensory cues, the data supporting this is indirect (mutants, RNAi) and relies on a single behavioral assay (reversal time). Other explanations for their data include neuropeptide signaling may alter development of these sensory cells or circuits and the behavioral changes may be secondary effects of underlying developmental defects. Indeed the authors report that RNAi treatments were done for two generations, and not post-developmentally, which makes this possibility more of a concern. As the paper appears now, it is probably more appropriate for a genetics journal. To increase interest of these findings and to strengthen the authors idea that neuropeptide secretion specifically (and not neuropeptide signaling generally) is relevant for this form of modulation, it is recommended that the authors directly examine the effects of octopaminergic signaling on neuropeptide secretion.

The following additional experiment is recommended:

To test the prediction that nlp-7 or nlp-8 are secreted from ADL in response to OA treatment, generate ADL specific nlp-7/8::GFP transgenic animals and assay nlp-7/8 secretion in response to acute octopamine treatment, ser-6 mutants, and in octopamine biosynthesis mutants by determining changes in 1)coelomocyte fluorescence and 2) DCV fluorescence in ADL compared to controls. This approach should be technically feasible because it has been successfully reported for ADL-secreted peptides (Ashrafi, PLoS Genetics, 2008). Alternatively, AWB/nlp-9 or ASH/nlp-3 could be examined in the same way.

Other comments:

Does nlp-8 overexpression have an effect on reversal time in che-36 mutants?

Figure 7C shows phenocopy of npr-15 mutants with RNAi of npr-15 using the nlp-1 promoter. Since both nlp-1 and npr-15 express in ASI and AWC, this experiment does not appear to distinguish the cell in which npr-15 functions. The gpa-4 promoter seems to be a better one for this experiment since it is ASI specific.

Figure 7C shows that the npr-18 phenotype can be generated using the flp-6 promoter. Flp-6 is expressed in ASE and several other neurons and npr-18 appears to be expressed in ASER and other cells in Figure 7A. The gcy-5 (or ceh-36) promoter, which is specific for ASE would be a better choice for this experiment to confirm function in ASE.

Figure 1 is missing the label G.

Expand table 7B to list ALL promoters used in study. Also specify which promoters were used in the text or figure legends. For example it is not clear which ADL promoter was used in Figure 5. Materials and methods suggests it would be sro-1 (which is ADL and SIA).

1st Revision - authors' response | 18 September 2011

Referee #1:

Major Comments:
1. "*Regarding SER-3 acting in ASH: Only sra-6::RNAi experiments are shown. Since this promoter also expresses in ASI and PVQ, a second promoter expressing in ASH but not ASI and PVQ should also be used to show that ASH is the relevant cell for RNAi phenocopy. An off-target control using a*

promoter not expressed in ASH should also be provided as a negative control for RNAi spreading. Finally, to test sufficiency as well as necessity of ASH expression for SER-3 modulation, cell-specific rescues of the ser-3 mutant should be performed in ASH, with an off-target control again provided."

We agree. We have included a second ASH promoter for the ser-3RNAi knockdown (*srb-6*) and have included ASH rescue data using both the *sra-6* and *srb-6* promoters. These data fully support our initial observations. In addition, have completely redesigned Figure 1. to include more control data, including an off target *ser-3* RNAi at 10 mM octanol.

2. *"To understand the role of SER-6 in ASH modulation, more work controls need to be provided to establish a cellular site of action. Since all transgenic lines in the paper rescue the mutant and all RNAi lines affect the behavior, it is difficult to say conclusively that these are the critical cells. At a minimum it is important to provide negative controls (for example using promoters whose expression does not overlap with the cells giving rescue/RNAi phenocopy) to show specificity for these effects."*

We agree. We routinely express the RNAi constructs in a number of neurons as a control for potential spreading. Neuron-selective RNAi has the advantage over rescue in that it requires the expression of the native receptor to be effective, once the potential for spreading has been eliminated. An "off-target" *ser-6*RNAi (using the *odr-2(2b)* promoter) has now been included in Figure 3.

In contrast, we have observed potential" off-target" phenotypes with the expression of many *C. elegans* G-protein coupled receptors, presumably because the ligands for the receptors are either tonically released or the receptors themselves exhibit constitutive activity in the absence of ligand. The effects of G-protein signaling on neurotransmitter release in *C. elegans* are well documented and we have observed that the expression of many G_s, G_o and G_q -coupled receptors in interneurons or motorneurons modulating locomotion, often yield artefactual locomotory phenotypes, just as the expression of the gain-of function G-proteins themselves. For example, the Bargmann lab has recently demonstrated that 2-fold changes in the expression level of tyra-3 can have profound effects on locomotry behavior (Bendesky et al., 2011). These observations make our use of both neuron-selective RNAi knockdown in conjunction with rescue even more relevant.

3. "In figure 3, the authors use eat-4RNAi to infer that glutamate is not required for the ASI/AWB effects. Have they done this experiment with unc-13RNAi, as they did for the ADL neurons? This would seem a more definitive way to test the requirement for synaptic vesicle transmission."

We understand this criticism. Certainly, other potential glutamate transporters are present in the *C. elegans* genome. Indeed, the negative result with the ADL::*eat-4*RNAi assumes that the knockdown was effective, which is problematic in the absence of a phenotype. In addition, recent publications suggest that although UNC-31 is required for DCV release, UNC-13 may play a role in both SV and DCV release. In fact, some of our unpublished work supports this observation. Therefore, to avoid confusion we have removed any of the *unc-13* data from the MS. In addition, since we have no direct evidence that the *eat-4*RNAi is actually effective in these experiment (i.e., no phenotype) we have also removed the *eat-4* data (and any other unconfirmed negative RNAi data).

4. *"In determining the site of action for npr-15, the authors conclude that npr-15 must act in AWC based on the nlp-1::npr-15RNAi phenotype, though nlp-1 is expressed in at least 10 different classes of neurons. The authors assume that npr-15 can only act in the cells where they observed expression of their GFP, but this puts too much faith in reporter transgene, even a rescuing one. To conclude that AWC is the site of action, they need to use a least one other independent promoter that expresses in AWC but not the other cells in the nlp-1 expression domain."*

We agree completely. We have added additional data using the *ceh-36* promoter (AWC and ASE) for both *npr-15*RNAi knockdown in wild type animals and rescue in *npr-15* null animals. As noted above, we have observed "off target" expression phenotypes with the expression of many G-protein coupled receptors, presumably because ligands for the receptors are either tonically released or the receptors themselves exhibit constitutive activity in the absence of ligand. Therefore, we feel much more confident confirming our rescue data by the neuron-selective RNAi knockdown of the native receptors using multiple promoters and taking care to assess for any potential RNAi spreading.

"As with other rescue experiments, they also need to include at least one off-target RNAi transgene

as a control."
We agree. Figure 7 includes three "off-target" *npr-15* transgenes as "off-target" controls *(glr-1*, *nmr-1* and *npr-9*)

5. *"The authors also state that ASE has been shown to be involved in attraction but not avoidance. However, activation of ASER (the neuron expressing npr-18) has been shown directly to evoke avoidance behavior, not suppress it (Suzuki, Nature 2008). Thus, enhancement of ASER activity would be predicted to enhance avoidance rather than attraction. Note that ASE has been implicated in avoidance behavior to CO2 (Bretscher, Neuron 2011)."*

We agree completely with the statements of the reviewer. In fact, these observations agree well with some of our recent data suggesting that NPR-18 signaling actually inhibits ASER. We have modified the text and the model accordingly. Our recent observations suggest that many sensory neurons are tonically active and the sum of their inputs dictates a variety of locomotory behaviors, i.e., reversal can be potentiated by stimulating aversive inputs or inhibiting inputs favoring continued forward movement (attraction in a simple sense). We thank the reviewer for these insightful comments.

6. "*There is quite a bit of data (for example, nlp-7;ser-6 and nlp-9,ser-6 double mutants) that is mentioned but not shown. Much of this includes controls that are important for the paper. These results should be provided, at the very least as supplemental figures."*

We agree. Data on ser-6;*nlp-7, ser-6;nlp-9* and *nlp-9;nlp-7* double mutants is now included. In addition, we have included the data on *nlp-7* and *nlp-9* overexpression in *ser-6* null animals, which we think may be what the reviewer is referring to.

7. "Essentially all the experiments in this paper look at effects of exogenous octopamine. Are any of the effects of receptor mutants mimicked by octopamine-deficient mutants? It might be useful to have a little more discussion about the potential biological significance of the modulatory pathways described here."

Interesting suggestion. Starvation for 50 min decreases aversive responses to 100% octanol and this decreased response is reduced in *tdc-1* and *tbh-1* null animals that would be predicted to have reduced OA levels. We have included this data in the Supplementary section.

Minor points:

1. Nomenclature: In all figures, overexpression lines should be labeled consistently (instead of using different labels like ser-6::ser-6, ser-6+, XS). Explain in figure legends what '+' and 'XS' means.

The figure legends have been modified accordingly and now include information on the black, gray and hatched bars.

2. Also, for transgenic rescue experiments, the actual promoter should be stated in the figure legend, rather than using constructions such as "ASI::npr-15RNAi". This is particularly true for cases like "ASH::ser-3RNAi" which are not precisely cell-specific. (It is also not accurate to refer to the sro-1::ser-6 transgene as "ADL::ser-6" since it is also expressed in the SIA neuron.)

Corrected

3. Finally, although nomenclature for gene fusions is not completely consistent in the literature, I think the use of constructions like pser-3::ser-3::gfp is confusing. Since four-letter gene names are now in use, pser-3 looks like it refers to a different gene from ser-3. According to http://wiki.wormbase.org/index.php/UserGuide:Nomenclature, the recommended usage for gene all gene fusions is, for example, ser-3::GFP , with an explanation in the legend if necessary to distinguish transcriptional from translational fusions.

Changed

4. *Discussion of the literature: There has been a lot of work on avoidance behavior in C. elegans, and the authors' discussion of this background seemed somewhat narrow. For example, there have been at least three studies published recently by other groups describing monoamine and/or neuropeptide modulation of ASH in response to food (Ezak, PlosOne 2010; Ezcurra, EMBO 2011; Singh, Curr Biol 2011). It is strange that none of these are cited, especially since they support the authors' point that ASH modulation is complex.*

We agree, the Ezak and Ezcurra studies are now referenced in the text.

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4. For clarity, please specify in text or figure legend which cells are expressing GFP in fig 3D.
Corrected

Referee #2 :

Major Issues

"What is the AWC-specific promoter for eat-4 knockdown (Fig 7D)? It would be more convincing if this promoter was used to rescue or knockdown npr-15 and thereby confirm it functions in AWC. Similarly, why not use the gcy-5 promoter to knockdown npr-18 expression specifically in ASER?"
The *eat-4* data have been removed, as discussed above. Interestingly, we have not had success using the *gcy-5* promoter for RNAi knockdown for reasons that are not clear. We have made similar observations for other neuron-selective promoters. Perhaps, their expression is too low or too late in development for RNAi to be effective.

"Seeing as your measure is 'time to reverse', it is essential to know how your genetic manipulations affect spontaneous reversal rate. Without this data, it is unclear if your effects were caused by changes in octanol sensitivity or just changes in basal locomotion. For each strain, it would be informative to have a negative control (ie 0% octanol), so readers have an idea of the spontaneous reversal rate of your animals."
We agree completely. Large changes in the rate of spontaneous reversal could have a potential to affect our assay. To evaluate this potential caveat, all animals are initially assayed in the absence of octanol, but given our assay conditions (assay limited to 20 sec) spontaneous reversals are only rarely observed. In contrast, in these assays, we do observe a limited number of animals that do not respond to octanol within 20 secs, presumably either because the hair was not properly loaded and stochastic variation in the animals being assayed. We have analyzed the data including or excluding these 20 sec animals and find no significant difference in the results.

One assumption throughout the paper is that the response to 100% octanol is ASH-mediated, ie "SER-6 functions in the ADL, AWB and ASI sensory neurons to inhibit ASH-mediated aversive responses." Chao et al. (2004) found that worms without ASHs responded "reasonably well" to 100% octanol off of food. Therefore your responses of interest may not actually be driven by ASH. This needs to be addressed early on in the paper. In figure 3E, it would be informative to see ASH eat-4 and egl-3 knockdown strains. Knockdown of eat-4 would presumably mostly silence the ASH component of the worm's response to 100% octanol.
An interesting observation that we have pondered extensively. We have examined animals expressing G-CaMP in either the ASHs, AWBs or ADLs. Octanol initiates a robust calcium signal in the ASHs, but has no effect on G-CaMP fluorescence in the AWBs or ADLs. We interpret these data to indicate either that the ASHs are the primary octanol sensor and that the AWBs and ADLs do not directly respond to octanol and instead may function downstream of an as yet unidentified octanol-sensing neuron or that ablation of the ASHs causes compensatory changes in the octanol responsiveness of the AWBs and ADLs. These calcium imaging data are now included in the text.

Minor comments

1. Page 8, line 1: The text cites: "Figures 1E-G", but there is no figure 1G.
Corrected

2. Page 9, line 8: The text states that "OCTR-1 does not appear to be involved in the OA inhibition of aversive responses to 100% 1-octanol," but figure 2a shows that octr-1 mutants respond more quickly than wild-type in the presence of 10mM OA. Please explain.
Discussed fully in the text. SER-3 antagonizes the OCTR-1-mediated inhibition

*2. Page 11, line 14: The text states: "Conversely, the expression of ser-6 in the ADLs, AWBs or ASIs of wild type animals inhibited basal aversive responses off food." From the graph and the statistical comparison (ie ** = different from wild-type in the presence of OA), it seems that expression of ser-6 in ADL and AWB did NOT inhibit basal aversive responses.*
Corrected

4. Page 29, line 8: "Similarly, in C. elegans, OA has no effect on basal aversive responses to submaximal ASH stimulation" Doesn't Figure 1a show that it does? N2 tested in the presence of OA and 5-HT respond slower than worms tested n the presence of 5-HT alone.

Discussion expanded. OA inhibits 5-HT stimulation, but not basal aversive responses to 30% octanol.

5. How does ser-6, eat-4, and egl-3 knockdown (Fig 3E) affect 100% octanol responses in the absence of OA?

Data on *ser-6* and *egl-3* now included.

Referee #3:

1. The main concern with this paper is that the results may be of limited interest for publication in EMBO for two reasons: First, the idea that neuropeptide signaling regulates aspects of sensory behavior has been well established by the previous work of the authors and several other groups using the C. elegans model.

We disagree. The observation that OA activates essential, more global multipeptide signaling cascades is novel, fully supported by the data in the MS and also suggests that the model system may be of potential utility in understanding the adrenergic modulation of pain in mammals.

2. *Several studies have gone on to directly show sensory invoked calcium changes in specific neurons, neuropeptide-receptor interactions, etc. Second, although the authors propose that specific neuropeptides are secreted from specific cells in response to sensory cues, the data supporting this is indirect (mutants, RNAi) and relies on a single behavioral assay (reversal time). Other explanations for their data include neuropeptide signaling may alter development of these sensory cells or circuits and the behavioral changes may be secondary effects of underlying developmental defects. Indeed the authors report that RNAi treatments were done for two generations, and not post-developmentally, which makes this possibility more of a concern. As the paper appears now, it is probably more appropriate for a genetics journal. To increase interest of these findings and to strengthen the authors idea that neuropeptide secretion specifically (and not neuropeptide signaling generally) is relevant for this form of modulation, it is recommended that the authors directly examine the effects of octopaminergic signaling on neuropeptide secretion.*

Certainly, these criticisms could apply to almost any *C. elegans* work. However, the present study has exploited multiple experimental approaches that combine both neuron-selective RNAi knockdown and rescue that significantly strengthen the conclusions presented in the MS.

The following additional experiment is recommended:

3. To test the prediction that nlp-7 or nlp-8 are secreted from ADL in response to OA treatment, generate ADL specific nlp-7/8::GFP transgenic animals and assay nlp-7/8 secretion in response to acute octopamine treatment, ser-6 mutants, and in octopamine biosynthesis mutants by determining changes in 1)coelomocyte fluorescence and 2) DCV fluorescence in ADL compared to controls. This approach should be technically feasible because it has been successfully reported for ADL-secreted peptides (Ashrafi, PLoS Genetics, 2008). Alternatively, AWB/nlp-9 or ASH/nlp-3 could be examined in the same way.

We agree that the direct OA stimulation of peptide release would strengthen the manuscript. However, the assay that the reviewer describes is based on the chronic, not the acute accumulation of venus in coelomocytes from animals overexpressing a tagged neuropeptide (the same criticisms outlined above for some aspects of our work). Indeed, these tagged peptides were expressed in multiple neurons that included the ADLs. We have been unable to demonstrate an acute effect of OA (after 2 hr incubation) on coelomocyte GFP accumulation in animals expressing either nlp-7::GFP or nlp-9::GFP. This result is probably not surprising, as the temporal dynamics of GFP coelomocyte accumulation have never been described and recent work suggests that, at least some, tagged neuropeptides do not accumulate in the coelomocytes at all (Hu et al., 2011 Neuron 71:92-102). Indeed, in our hands, different coelomocytes in the same animals give different and sometimes conflicting levels of GFP accumulation. In addition, it is clear from work in mammals and other invertebrate systems, that the addition of tag may cause at last some of the peptides to localize differently from native peptides identified by immunolocalization. Indeed, even though

both of our tagged constructs rescued null animals, the nlp-7::GFP appeared punctate and accumulated in coelomocytes, the nlp-9::GFP was diffuse and did not accumulate. It would have been gratifying if these studies had demonstrated a direct acute effect of OA on peptide release, but the absence of an observed effect in these assays does not invalidate our proposed hypothesis, given the number of potential experimental artifacts in the assay system.

Other comments:

1. Does nlp-8 overexpression have an effect on reversal time in che-36 mutants?

Good idea. As predicted, it does not.

2. Figure 7C shows phenocopy of npr-15 mutants with RNAi of npr-15 using the nlp-1 promoter. Since both nlp-1 and npr-15 express in ASI and AWC, this experiment does not appear to distinguish the cell in which npr-15 functions. The gpa-4 promoter seems to be a better one for this experiment since it is ASI specific.

As noted above, we have added additional promoters and rescue to the data that support our initial conclusions.

3. Figure 7C shows that the npr-18 phenotype can be generated using the flp-6 promoter. Flp-6 is expressed in ASE and several other neurons and npr-18 appears to be expressed in ASER and other cells in Figure 7A. The gcy-5 (or ceh-36) promoter, which is specific for ASE would be a better choice for this experiment to confirm function in ASE.

We have discussed the *gcy-5* promoter above.

4. Figure 1 is missing the label G.

Corrected

5. Expand table 7B to list ALL promoters used in study. Also specify which promoters were used in the text or figure legends. For example it is not clear which ADL promoter was used in Figure 5. Materials and methods suggests it would be sro-1 (which is ADL and SIA).

Added to the supplement

2nd Editorial Decision 24 October 2011

Thank you for submitting your revised manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments are provided below. As you can see, the referees appreciate the introduced changes and support publication here. Referee #3 has two remaining points that would be nice to get resolved. I don't know if you have data on hand to address comment #1, if not then we can discuss this issue further.

We will proceed with the acceptance of the paper for publication here once we receive the revised version.

Best

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

I think the authors have done a very good job responding to reviewer comments. This is interesting work and I am in favor of publication in the EMBO Journal.

Referee #2

The authors have sufficiently responded to the comments. The authors have also done a good job of addressing the concerns of reviewer 1 - including all of the extra control groups was an important addition to the manuscript. I would disagree with reviewer 3 that the results are of limited interest for EMBO readers. Generally speaking, previous publications have reported the role of peptidergic signalling in avoidance of octanol, but this manuscript describes a novel mechanistic viewpoint. The additional experiments proposed by reviewer 3 could further support the data, but are technically demanding and may not lead to definitive answers.

Referee #3

The authors sufficiently addressed many concerns and have attempted to visualize secretion of tagged neuropeptides without success. The paper is much stronger now with the addition of controls for spreading and cell specificity, and the work represents a significant advance in our understanding of the involvement of neuropeptide signaling in regulating behavioral circuits. Thus, we recommend this paper for publication if the following two concerns are addressed.

1) In Figure 3, experiments using the odr-2 promoter were added to control for off target effects of RNAi. It would be important to show that this promoter is functional in the authors hands to validate it as a control for spreading. Alternatively the authors could use a promoter that has already validated in this study. For example the ceh-36 promoter (ASE, AWC) could be used as an off target control for ser-6 RNAi in Figure 3E, since it was shown to be functional in Figure 8A.

2) Because multiple promoters with complex expression patterns are used, it would be helpful if the cells that each promoter expresses in were listed in either the text or the figure legends so that readers don't have to go to the supplemental chart to interpret the figures.

2nd Revision - authors' response 25 October 2011

We would like to thank you and referees for their positive comments. The two criticisms outlined by referee 3 have been addressed directly.

1. In Figure 3, experiments using the odr-2 promoter were added to control for off target effects of RNAi. It would be important to show that this promoter is functional in the authors hands to validate it as a control for spreading. Alternatively the authors could use a promoter that has already validated in this study. For example the ceh-36 promoter (ASE, AWC) could be used as an off target control for ser-6 RNAi in Figure 3E, since it was shown to be functional in Figure 8A.

We have used the *odr-2(2b)* promoter in previous publications to demonstrate a role for *mod-1* in the 5-HT stimulation of aversive responses (Harris et al., 2009, J. Neuroscience 29:1446), validating the use of this promoter in our hands.

2. Because multiple promoters with complex expression patterns are used, it would be helpful if the cells that each promoter expresses in were listed in either the text or the figure legends so that readers don't have to go to the supplemental chart to interpret the figures.

We list of cells expressing the individual promoters has now been incorporated into the text (Methods section), as suggested by the referee.

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