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Hypoxia Regulates Glutamate Receptor Trafficking through a HIF-Independent Mechanism

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 16 September 2011

Thank you for submitting your manuscript entitled for consideration by the EMBO Journal.
As you will see, we share the referees interest in the dataset and see high potential for a successful revision.

I must apologize for the excessive delay. Unfortunately, it took an atypical number of requests to assemble an informed referee panel, and, as it turns out, one of the referees is not delivering his/her report in a timely manner. We tried hard to obtain this report, since the report of ref 2, while positive, is not as detailed as we expect at this journal, somewhat undermining the weight we can give this report.

Referee 1, who sent the constructive evaluation appended to this letter, would like to see two key experimental improvements:
1) biological validation of additional double mutant and rescue strains using the established behavioral analysis
2) biochemical demonstration that lin-10 is hydroxylated on specific prolines by egl-9
In addition, the referee suggests additional analysis of the egl-9 cdk-5 strain.

Regarding point 5: it is actually this journal's explicit policy to encourage citation of the primary literature over reviews. 20 primary papers is of course a tall order and it is certainly legitimate to reduce primary citations to key papers, and to add a general review to overview the bigger picture.

Given the referees' positive recommendations, I would like to warmly invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is

EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

We will only return an adequately revised manuscript to referee 1, who delivered a constructive report rapidly, so there should be minimal delay in the next round.
If we receive a report from referee 3 (who has been chased multiple times already) during revision, we will forward it. We would only expect that real caveats missed by the current referees would have to be addressed. This referee will not be chased further at this point, but it is in everyone's interest that essential points that may in principle emerge are still considered.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let us know in advance and we should be able to extend the revision period.

Thank you for the opportunity to consider your work for publication and your considerable patience in awaiting this decision. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This manuscript proposes an interesting new model for hypoxia regulation of neuronal function: that the prolyl hydroxylase EGL-9 of C. elegans modulates glutamate receptor traffic in hypoxic conditions through a direct interaction with LIN-10 (mint), not via the standard HIF-1 transcriptional mechanism. The results in the paper show that (1) egl-9 mutations and hypoxia affect the distribution of GFP-tagged glutamate receptors (2) egl-9 mutations and hypoxia affect reversal behaviors in a similar fashion as glutamate receptor glr-1 mutations (3) egl-9 mutations affect glr-1-mediated currents in C. eelgans neurons (4) the relevant egl-9 isoform, egl-9E, is localized at synapses (5) the lin-10 scaffolding protein binds egl-9 and is required for it effects on GFP-tagged glutamate receptor traffic (6) egl-9 and the cyclin-dependent kinase cdk-5 have opposite effects on GFP-tagged glutamate receptors, and cdk-5 is epistatic to egl-9.

The strength of the paper is the novelty of the hypothesis. Hypoxia and glutamate receptors are both subjects of great interest in the nervous system. Bringing these two together in a novel signaling mechanism could have a large impact,

The weakness of the paper is that many results are obtained only through visual analysis of GFP-tagged receptors, which are an indirect reporter of biological activity. Two areas that would strengthen the conclusions are (1) further biological validation of the model using behavioral analysis and (2) biochemical demonstration that lin-10 is modified by egl-9, which is suggested but not tested.

1. Biological validation. The two direct measurements of glutamate receptor function are a reversal assay (Figure 2a) that is applied to a subset of genotypes and an electrophysiological assay (Figure

2c-d) that is applied to only one genotype. The other measurements are GFP puncta that can represent many different active and inactive pools of proteins. The paper would be greatly strengthened by examining more of the double mutant and rescue strains that already exist in the behavioral assay used in Figure 2A.

What is the phenotype of an egl-9 glr-1 double mutant? Predicted = same as single mutants. What is the effect of hypoxia on an egl-9 glr-1 double mutant and on an egl-9 lin-10 double mutant? Predicted = no effect. What are the behavioral effects of the glr-1::egl-9E rescue transgene from Fig 3E? Predicted = full rescue of reversals.

It would also be interesting to examine egl-9 cdk-5 and lin-10 cdk-5, but the predictions are not as clear since cdk-5 has other targets.

2. Evidence for hydroxyprolylation of LIN-10 protein would be extremely valuable. Have the authors tried anti-hydroxyproline antisera in IP-Western blots? Is there any evidence for modification based on protein mobility (Figure 5I)? I would not consider a positive result to be a condition of publication, but an exploration of this direction could take the paper to the next level.

Minor points:
3. The effect of hypoxia on lin-10 should be examined in the egl-9 mutant and in the egl-9 cdk-5 double mutant (as in Figure 5G). Predicted = no effect.

4. The egl-9 cdk-5 double mutant should be tested for its effects on lin-10 localization to determine whether the epistasis relationship is the same as for glr1 localization.

5. 20 references for one general point in the first paragraph is overkill. Cite a review.

6. Page 7 describes rescue results with egl-9E and egl-9C, doesn't mention results with egl-9A which also fails to rescue (Figure 3C). Would be clearer to mention all three.

Referee #2 (Remarks to the Author):

The authors decribe a novel hypoxia-triggered pathway that controls glutamate receptor activation. This is a very nice paper, the experiments are well thought through, well executed and clearly described. I find the conclusions novel and interesting and recommend publication in EMBO J. without hesitation.

1st Revision - authors' response 25 November 2011

<u>Reviewer #1</u>

This manuscript proposes an interesting new model for hypoxia regulation of neuronal function: that the prolyl hydroxylase EGL-9 of C. elegans modulates glutamate receptor traffic in hypoxic conditions through a direct interaction with LIN-10 (mint), not via the standard HIF-1 transcriptional mechanism. The results in the paper show that (1) egl-9 mutations and hypoxia affect the distribution of GFP-tagged glutamate receptors (2) egl-9 mutations and hypoxia affect reversal behaviors in a similar fashion as glutamate receptor glr-1 mutations (3) egl-9 mutations affect glr-1-mediated currents in C. eelgans neurons (4) the relevant egl-9 isoform, egl-9E, is localized at synapses (5) the lin-10 scaffolding protein binds egl-9 and is required for it effects on GFP-tagged glutamate receptor traffic (6) egl-9 and the cyclin-dependent kinase cdk-5 have opposite effects on GFP-tagged glutamate receptors, and cdk-5 is epistatic to egl-9.

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The weakness of the paper is that many results are obtained only through visual analysis of GFP-tagged receptors, which are an indirect reporter of biological activity. Two areas that would strengthen the conclusions are (1) further biological validation of the model using behavioral analysis and (2) biochemical demonstration that lin-10 is modified by egl-9, which is suggested but not tested.

1. Biological validation. The two direct measurements of glutamate receptor function are a reversal assay (Figure 2a) that is applied to a subset of genotypes and an electrophysiological assay (Figure 2c-d) that is applied to only one genotype. The other measurements are GFP puncta that can represent many different active and inactive pools of proteins. The paper would be greatly strengthened by examining more of the double mutant and rescue strains that already exist in the behavioral assay used in Figure 2A.

We thank the reviewer for this insightful assessment of the paper, and for constructive and quite helpful comments. We agree that a more extensive behavioral analysis would provide biological validation for our argument. We therefore expanded our behavioral analysis by examining two well-characterized and published behavioral modalities that are mediated by GLR-1-containing AMPARs in *C. elegans*. First, we expanded our examination of reversal behaviors that we presented in the initial draft of the manuscript, as requested by the reviewer. The frequency of spontaneous reversals in direction of locomotion during foraging is directly proportional to the level of AMPAR signaling in the command interneurons of *C. elegans*. Second, we performed nose touch mechanosensation assays, which we did not include in the initial draft. Nose-touch assays allow us to analyze the synaptic efficacy between the ASH mechanosensory neuron and its interneuron targets, which express GLR-1 AMPARs. The frequency with which animals reverse direction upon presentation of a mechanosensory stimulus is directly dependent on the levels of AMPAR signaling between ASH and the command interneurons. The nose-touch assay is particularly useful for discriminating small differences in AMPAR function when such function is only partially impaired. The experimental findings from these two behavioral modalities correlate well with each other and with the GLR-1 cell biological and electrophysiological data presented in our initial draft. We have added this new data as Figures 2A, 2B, 3D, 4M, 4N, 6L, 6M, and S1G.

What is the phenotype of an egl-9 glr-1 double mutant? Predicted = same as single mutants.

We have generated the *egl-9 glr-1* double mutant and have found, as the reviewer predicted, that it has a similar (but not stronger) effect on GLR-1-mediated behaviors as observed for the single mutants. This data has been added as Figure 2A,B.

What is the effect of hypoxia on an egl-9 glr-1 double mutant and on an egl-9 lin-10 double mutant? Predicted = no effect.

We have also generated the *egl-9 lin-10* double mutant and have found, as the reviewer predicted, that it has a similar (but not stronger) effect on GLR-1-mediated behaviors as observed for the single mutants. For both *egl-9 glr-1* and *egl-9 lin-10* double mutants, there is no added effect of hypoxia treatment, as the reviewer predicted. The data for the *egl-9 glr-1* double mutants has been added as Figure 2A,B. The data for *egl-9 lin-10* double mutants has been added as Figure 4M,N.

What are the behavioral effects of the glr-1::egl-9E rescue transgene from Fig 3E? Predicted = full rescue of reversals.

We have examined the reversal behavior of *egl-9* mutants rescued with the P_{glr-1}*::egl-9E* transgene. As predicted by the reviewer, we observed full rescue of the reversal behavior. This data has been added as Figure 3D.

It would also be interesting to examine egl-9 cdk-5 and lin-10 cdk-5, but the predictions are not as clear since cdk-5 has other targets.

This is an excellent experiment. We have generated the *egl-9 cdk-5 and lin-10 cdk-5* double mutants and analyzed their behavior. We find that the reversal frequency and nose touch sensory behavior of *egl-9 cdk-5* double mutants is restored to wild-type levels. Thus, CDK-5, while not having a dramatic role in regulating GLR-1 under normoxic conditions, is essential for the changes in GLR-1 trafficking and GLR-1-mediated behaviors observed in both hypoxic animals and in *egl-9* mutants. We also examined the reversal frequency and nose touch sensory behavior of *lin-10 cdk-5* double mutants. We found that the behaviors of these double mutants resembled those of *lin-10* single mutants. Thus, LIN-10 is essential for the changes in GLR-1 trafficking and GLR-1-mediated behaviors observed in *cdk-5* mutants. Taken together, this genetic epistasis data indicates that EGL-9 and oxygen act upstream of CDK-5, whereas LIN-10 acts downstream of CDK-5. This finding strongly supports our hypothesis, and we have included it as new data in Figure 4M,N and Figure 6L,M.

2. Evidence for hydroxyprolylation of LIN-10 protein would be extremely valuable. Have the authors tried anti-hydroxyproline antisera in IP-Western blots?

We agree and have tried a number of approaches to test this idea. First, we attempted to test whether EGL-9 hydroxylates LIN-10 *in vitro*. Unfortunately, we have not been able to produce soluble recombinant EGL-9. Expression from bacterial vectors of either full length EGL-9 cDNAs or shorter truncations of the EGL-9 catalytic domain produce insoluble protein regardless of the different bacterial expression strains that we have tried. We have tried expressing recombinant EGL-9 protein in cultured cells, but have found it to be highly unstable. We have tried to produce it using *in vitro* translation commercial kits, but again have found it to be unstable even in these kits, even if we add critical cofactors (e.g., iron, oxoglutarate).

As an alternative approach, we tried the reviewer's excellent suggestion of testing the commercial anti-hydroxyproline antisera through Western blots. We generated lysates from wild-type animals lacking transgene (a negative control), wild-type animals containing the *odIs22[LIN-10::GFP]* transgene, *egl-9(sa307)* mutants containing the *odIs22[LIN-10::GFP]* transgene, and *cdk-5(ok626)* mutants containing the *odIs22[LIN-10::GFP]* transgene. Using anti-GFP antibodies, we could successfully pull down LIN-10::GFP protein from lysates and detect it by Western blot from wild-type and mutant animals containing the transgene, but not from wild-type animals lacking the transgene. However, we could not successfully detect any signal using commercially available anti-hydroxyproline antibodies, even when we varied antibody titration, binding times and conditions, and blocking buffers. The only published Western blot for this antibody comes from Jung et al. *Tissue Engineering Part A* (2011), in which the antibody is used to detect collagen in human periodontal ligament stem cells. These cells are rich in collagen, and hydroxyproline residues are highly abundant in individual collagen molecules (about 10% of the total collagen protein sequence is hydroxyproline), which could explain the ease with which it was detected using commercial anti-hydroxyproline antibodies. By contrast, we would only expect a handful of hydroxyproline residues per individual molecule of LIN-10; it's quite possible that the antibody lacks the sensitivity to detect such low levels. Thus, our results with the anti-hydroxyproline experiments neither prove nor disprove our hypothesis. We have therefore opted not to include them in our manuscript.

Is there any evidence for modification based on protein mobility (Figure 5I)?

This is also an excellent suggestion. Unfortunately, the size of LIN-10 protein as resolved by SDSPAGE is around 150 kDa. At this large size, it is difficult to detect small deviations in electrophoretic mobility resulting from covalent modification of amino acid side chains. Nevertheless, we have tried to observe such changes using transgenes in which either the prolines or the serines/threonines on LIN-10 have been mutated to alanines. We have not detected significant shifts in mobility for these mutant LIN-10 proteins, either with standard SDSPAGE or with SDSPAGE coupled with the PhosTag reagent, which retards the mobility of phosphorylated

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proteins. Given the difficult nature of this experimental approach, our lack of findings here neither support nor rule out our hypothesis.

I would not consider a positive result to be a condition of publication, but an exploration of this direction could take the paper to the next level.

We agree that a demonstration of LIN-10 hydroxylation by EGL-9 would provide additional biochemical support for our hypothesis. Nevertheless, we believe that our genetic, behavioral, and cell biological data provide a compelling case that EGL-9 works through its regulation of LIN-10. EGL-9 binds to, colocalizes with, and directs the subcellular localization of LIN-10. Chemical agents that remove iron from the catalytic core of EGL-9 mimic the effects of *egl-9* mutations on GLR-1 trafficking. Similarly, an amino acid substitution within the catalytic core of EGL-9 also mimics the effects of *egl-9* mutations on GLR-1 trafficking. Thus, EGL-9 catalytic activity as a prolyl hydroxylase does seem to be a critical component of GLR-1 regulation. Finally, while we have not shown that EGL-9 directly hydroxylates the amino-terminal prolines found on LIN-10, we have shown that site directed mutagenesis of these same prolines blocks the effect of both hypoxia and *egl-9* mutations *in vivo*. We have added a sentence to the end of the Results section to highlight that other models beside direct hydroxylation are possible.

Minor points:
3. The effect of hypoxia on lin-10 should be examined in the egl-9 mutant and in the egl-9 cdk-5 double mutant (as in Figure 5G). Predicted = no effect.

We have examined LIN-10 subcellular localization in both *egl-9* single mutants and *egl-9 cdk-5* double mutants under conditions of hypoxia. Similar to *egl-9* single mutants under normoxia, *egl-9* single mutants under hypoxia have few LIN-10 puncta along their ventral cord dendrites. Similar to *egl-9 cdk-5* double mutants under normoxia, *egl-9 cdk-5* double mutants under hypoxia have wild-type levels of LIN-10 puncta along their ventral cord dendrites. We have included this new data in Figure 5G,H.

4. The egl-9 cdk-5 double mutant should be tested for its effects on lin-10 localization to determine whether the epistasis relationship is the same as for glr1 localization.

We have examined LIN-10 subcellular localization in *egl-9 cdk-5* double mutants under conditions of hypoxia. Mutations in *cdk-5* can block the effects of *egl-9* mutations (and hypoxia treatment) with respect to LIN-10 subcellular localization (Figure 6F,G,H). This matches the epistasis relationship observed using GLR-1 localization as a phenotypic output, as mutations in *cdk-5* can block the effects of *egl-9* mutations (and hypoxia treatment) with respect to GLR-1 subcellular localization (Figure 6E,K,L,M).

5. 20 references for one general point in the first paragraph is overkill. Cite a review.

We apologize for overzealous citations in the first paragraph. We have reduced the number of citations by focusing on a few key papers.

6. Page 7 describes rescue results with egl-9E and egl-9C, doesn't mention results with egl-9A which also fails to rescue (Figure 3C). Would be clearer to mention all three.

We have now mentioned our findings with regard to EGL-9A on this page to make this section more clear.

Reviewer #2

The authors decribe a novel hypoxia-triggered pathway that controls glutamate receptor activation. This is a very nice paper, the experiments are well thought through, well executed and clearly

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described. I find the conclusions novel and interesting and recommend publication in EMBO J. without hesitation

We are glad that the reviewer found our paper to be convincing and interesting, and thank the reviewer for his or her enthusiastic review.

2nd Editorial Decision 23 December 2011

I am very pleased to inform you that your interesting manuscript has been accepted for publication in the EMBO Journal, pending the minor textural clarification suggested by the referee (see report below).

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

The revised version is significantly improved and appropriate for publication.

A couple of minor comments:

I think most readers would assume that "hypoxia" represents animals that are actually observed in hypoxia. However, the protocol used in this paper is treatment with 24 hours of hypoxia followed by 12 hours of normoxia, a more complicated set of manipulations. The authors should describe this protocol when they first introduce the experiments on page 5. It would also be useful to include additional information if it exists - when does the effect arise, how long does it persist.

Page 10. The description of cdk-5 makes it sound as if it phosphorylates proline. Change the wording to "the proline-directed serine-threonine kinase CDK-5" when it first appears. In the next sentence, say "phosphorylating multiple proline-proximal sites".

Page 11. I think there is an error on this page 8-9 lines from the bottom. It does not appear that the proline-substituted LIN-10::GFP transgene has increased punctate localization in normoxia. It looks like WT in Figure 7B,D,H.

Figure 4A legend. I think it is standard to refer to a phosphorylation site residue based on the serine or threonine, not the adjacent proline.

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