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Cis Interaction Between Semaphorin6A and Plexin-A4 modulate the repulsive response to Sema6A

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1st Editorial Decision 22 January 2010

Thank you for submitting your paper to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are enclosed below.

As you can see, the referees find the analysis interesting, well done and suitable for publication here. However, there are also some issues that need to be resolved before further consideration here. Some important controls are missing and some insight into the effects of the cis Sema6a/plexin-A4 interaction on plexinA4 downstream signaling is needed. Also the analysis concerning the domains involved in the cis and trans interaction needs to be improved. Should you be able to address the raised concerns, we would consider a revised manuscript. I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. 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 initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal
REFEREE REPORTS

Referee #1 (Remarks to the Author):

The authors investigate the co-expression of plexin4 with sema6 in the peripheral nervous system, which the authors show leads to a desensitization of these axons towards sema6. Sensory axons show this co-expression, and are insensitive to sema in trans, while sympathetic axons express only plexin4 and are sensitive towards sema6 in trans. Authors support their idea by outgrowth assays and collapse assays using material from knockouts of sema6A and/or plexinA4.

In established cell lines, they show that co-expression in cis of plexin and sema prevents binding of sema6A in trans. They show that the effect is not due to reduced surface expression of plexins. They map superficially the cis and trans interaction domains between semas and plexins, and show that even in the absence of the sema and the Ig domain there is a cis interaction between sema and plexins.

Comments:

The data are derived from in vitro assays, there are no in vivo data presented, although in some experiments tissue from knockout mice is used.

This manuscript represents a straight forward analysis of the meaning of a cis-interaction between semas and plexins and comes to a clear conclusion. Overall - also based on data from other groups - it appears that cis sema/plexin interactions are relevant in vivo, and represent one aspect of the sema/plexinA involvement in early neural development.

Block of trans binding: Is there the same amount of these proteins present on transfected cells? Western blot towards a quantification is necessary.

What is in turn the ratio of plexinA4 and sema6 on DRG cells and axons?

In the same direction reg. the growth cone collapse: It appears that the sensory growth cones indeed are sensitive to sema6A and removal of sema6A in cis increases to some extend, but not dramatically their sensitivity. What are the binding patterns here, e.g. does sema6A-Fc stain these axons?

An analysis of a signaling pathway downstream of plexinA4 would be of interest and should strengthen the view that cis-expression of sema6 downregulates or even completely abolishes the activation of the plexinA4 pathways.

At another level of analysis, overexpression of sema6A on sympathetic axons should lead to their desensitization. Do the authors observe this?

Referee #2 (Remarks to the Author):

The manuscript by Haklai-Topper and coworkers describes a novel and interesting finding, i.e. the functional relevance of the interaction in cis between transmembrane Semaphorin 6A (Sema6A) and its receptor Plexin-A4 co-expressed in certain neuronal cells. The Authors found that this interaction leads to a functional impairment of the receptor Plexin-A4, which becomes unable to interact with Sema6A presented in trans by neighbouring regulatory cells. The work is convincing. However, there are confusing parts that need clarification, and certain experiments require additional important controls, as discussed in the specific points below.

1) Figure 4. The Sema6A-PlexinA4 co-precipitation experiments shown in panel C need to be repeated including a specificity control, e.g. a control unrelated antibody for immuno-precipitations of Sema6A-transfected cells. Moreover, a confocal immunofluorescence analysis of the cells shown in panel B might demonstrate the co-clustering of the two molecules in discrete protein complexes on the cell surface.

2) Data in Figure 5A demonstrate that the binding of extracellular soluble Sema6A to surface receptor PlexinA4 is significantly inhibited by co-expression of Sema6A in target cells. However, to be conclusive, these experiments need an important control of the level of PlexinA4 on the cell surface, upon Sema6A co-expression. This should be done by surface antibody staining or by
surface protein biotinylation, followed by data quantification. Moreover, the data shown in Fig. 5B, seem to derive from different experiments and lack any loading control.

3) The last paragraph of Results sustains an important conclusion ("the cis interaction between Sema6A and Plexin-A4 requires different domains that those utilized for ligand binding in vivo"), which is weakly supported by experimental data. In fact the data shown in Figure 6 are partly confusing when compared to their description in the text, they lack some appropriate controls, and even the labelling in the figure seems to require revision. For example, labelling of panel C suggests that IPs were done with anti-Sema6A antibodies, and immunoblotted with anti-myc antibody, which would detect the same protein (since Sema6A is myc-tagged) and not the associated Plexin-A4 (which in other figures is reported to be Flag-tagged). Moreover, the manuscript text states that "cis binding was not abolished by deletion of either the Sema domain or the Ig domain of Plexin-A4 (Fig. 6B)", however none of the panels in Fig.6 contains these data. Results shown in panel C may rather suggest that the delta-sema mutant of Plexin-A4 has lost the ability to associate with Sema6A while the delta-Ig mutant is proficient in this (thereby different from that stated in the text). Notably, a control experiment by co-expressing Sema6A and full-size Plexin-A4 should necessarily be included here for comparison. Moreover, Fig. 6B shows that all Plexin-A4 extracellular domain mutants are unable to bind secreted Sema6A, but there is no evidence about the expression of these mutated receptors on the cell surface (which could be jeopardized by large protein truncations). In principle, if Plexin-A4 delta-sema mutant would not be expressed on the cell surface, it could not be able to bind any ligand in trans, but still associate in cis intracellularly with Sema6A co-expressed in the same cells. Unless all these confusing points are cleared out, it is impossible to draw compelling conclusions about this important structural and molecular aspect of the study.

Additional and minor points:

4) In the Discussion, the Authors raise a very intriguing issue that would deserve some analysis in the present work: is Sema3A signalling mediated by PlexinA4-NP1 complexes affected by the co-expression of Sema6A in the cells?

5) Pages should be numbered to facilitate reviewing work.

Referee #3 (Remarks to the Author):

This paper represents an important advance in the study of the functions and interactions of transmembrane semaphorins and plexins. These molecules have been shown through mainly genetic analyses to be involved in many important axon guidance and cell migration decisions in the developing mammalian nervous system. Phenotypic analyses across several mutants have shown however that the phenotypic congruence across mutants that would be expected of a simple ligand-receptor interaction is not present. There are in fact highly complex and context-dependent genetic interactions between mutations in Sema6A, Sema6B, PlxnA2 and PlxnA4. The nature of their biochemical interactions, which may underlie this complexity, has to date been studied only at a fairly superficial level.

The authors present the first detailed study of the biochemical interactions between two of these molecules, Sema6A and PlxnA4. It reveals the very interesting and important finding that the co-expression in cis of Sema6A in responding cells blocks the response of PlxnA4 to Sema6A in trans. Given that many of the responding populations of cells in vivo co-express some level of Sema6A, this suggests that this modulatory mechanism is highly relevant in many contexts. The fact that the cis interaction seems to involve distinct domains from the trans interaction also has implications for the molecular logic of the system.

The experiments are very convincingly done and the level of quality very high.

I have one suggestion for an additional experiment that might add to the conclusions. Given that PlxnA4 is a receptor for Sema6B as well it would be very interesting to know whether expression of Sema6A in cis blocks the binding of Sema6B in trans (and vice versa). If this is so then it means that the implications of the interaction in cis are broader and extend beyond the functions of just the two molecules involved. It also means that some of the phenotypes observed when Sema6A is deleted may be due to heightened responsiveness of PlxnA4 to Sema6B signals, which would still be present in this background. These data would thus be highly relevant to the interpretation of the phenotypes seen (and not seen) in the single mutants and also of the epistatic interactions that have
been observed in various double mutants in different parts of the brain. Addressing this point could add substantially to the impact of the paper.

1st Revision - authors' response 28 May 2010

Referee #1

The data are derived from in vitro assays, there are no in vivo data presented, although in some experiments tissue from knockout mice is used. This manuscript represents a straightforward analysis of the meaning of a cisinteraction between semas and plexins and comes to a clear conclusion. Overall - also based on data from other groups - it appears that cis sema/plexin interactions are relevant in vivo, and represent one aspect of the sema/plexinA involvement in early neural development.

Block of trans binding: Is there the same amount of these proteins present on transfected cells? Western blot towards a quantification is necessary.

We added controlled western blots of whole cell extract (Fig 5E) and of cell of surface biotinylation (Fig 5F), demonstrating that comparable amounts of Plexin-A4 are present in the transfected cells and on the cell membrane with or without the presence of Sema6A.

What is in turn the ratio of plexinA4 and sema6 on DRG cells and axons? In the same direction reg. the growth cone collapse: It appears that the sensory growth cones indeed are sensitive to sema6A and removal of sema6A in cis increases to some extent, but not dramatically their sensitivity. What are the binding patterns here, e.g. does sema6A-Fc stain these axons?

Unfortunately, there are no anti-Plexin-A4 antibodies that can be used to examine the amount of Plexin-A4 in DRGs. Thus, it is hard to determine the ratio of Plexin-A4 and sema6A. The functional receptor for Sema6A in these axons is Plexin-A4 since in its absence there is no collapsing response of sensory growth cones (Fig 3B). We believe that Sema6B, which is expressed in DRGs (Supplementary Figure 2) and (Suto et al., 2005) and can also act as cis inhibitor of Plexin-A4 (supplementary Fig 2) may account for the additional inhibition. Thus, future analysis of a Sema6A/ Sema6B double KO mouse, which is currently unavailable, may reveal a more robust enhancement in the response to Sema6A.

An analysis of a signaling pathway downstream of plexinA4 would be of interest and should strengthen the view that cis-expression of sema6 down regulates or even completely abolishes the activation of the plexinA4 pathways.

Our model suggests that Sema6A cis inhibition prevents ligand binding to Plexin-A4. Thus, we assume no signaling pathway downstream of Plexin-A4 is activated. It should be noted that the signaling pathways downstream of Plexin-A4 are largely unknown and currently there is no assay to test this in quantitative manner. The only available way to assess the activity is to examine changes in axonal growth-cones (Fig 3) or cell shape (as we did in supplementary Fig 4). However, the reviewer comment prompted us to test whether ligand independent activation of Plexin-A4 can be regulated by Sema6A.

We found the Sema6A can still interact to some extent in cis with Plexin-A4 that lacks the extracellular part (Fig 6D). This Plexin-A4 mutant is constitutively active, leading to the cellular contraction as shown in supplementary Fig 4 and in previous studies (Oinuma et al., 2004; Takahashi and Strittmatter, 2001; Turner and Hall, 2006). We found no inhibition of this Plexin-A4 mutant by co-expression of Sema6A (supplementary Fig 4). This observation, in combination with the fact that we did not find a requirement for the Plexin-A4 intracellular domain for cis interaction with
sema6A (Fig 6D), argues that that cis interaction regulates ligand binding and not a specific signaling pathway.

At another level of analysis, overexpression of sema6A on sympathetic axons should lead to their desensitization. Do the authors observe this?

Unfortunately we have not been able to establish a consistent expression system of Sema6A in embryonic sympathetic neurons, since the yield of this neuron type after dissection is very low.

Referee #2

The manuscript by Haklai-Topper and coworkers describes a novel and interesting finding, i.e. the functional relevance of the interaction in cis between transmembrane Semaphorin 6A (Sema6A) and its receptor Plexin-A4 co-expressed in certain neuronal cells. The Authors found that this interaction leads to a functional impairment of the receptor Plexin-A4, which becomes unable to interact with Sema6A presented in trans by neighbouring regulatory cells. The work is convincing. However, there are confusing parts that need clarification, and certain experiments require additional important controls, as discussed in the specific points below.

1) Figure 4. The Sema6A-PlexinA4 co-precipitation experiments shown in panel C need to be repeated including a specificity control, e.g. a control unrelated antibody for immuno-precipitations of Sema6A-transfected cells.

We provide new control co-precipitation experiments with anti-HA antibodies supporting the specificity of our co-precipitation experiments (Supplementary Fig 1). It should be noted that we also performed the co-precipitation experiments with anti-Sema6A antibodies and myc tagged Plexin-A4 (Fig 6D) (see below), which further supports the specific interaction of Plexin-A4 and Sema6A.

Moreover, a confocal immunofluorescence analysis of the cells shown in panel B might demonstrate the co-clustering of the two molecules in discrete protein complexes on the cell surface.

We now provide in addition to the cell surface staining also confocal immunofluorescence analysis, which supports the model that both Sema6A and Plexin-A4 co-reside on the cell membrane (Fig 4 C).

2) Data in Figure 5A demonstrate that the binding of extracellular soluble Sema6A to surface receptor PlexinA4 is significantly inhibited by co-expression of Sema6A in target cells. However, to be conclusive, these experiments need an important control of the level of PlexinA4 on the cell surface, upon Sema6A co-expression. This should be done by surface antibody staining or by surface protein biotinylation, followed by data quantification. Moreover, the data shown in Fig. 5B, seem to derive from different experiments and lack any loading control.

We now provide a new whole cell extract western blot (with loading control) demonstrating that the same amount of Plexin-A4 is present in the transfected cells (Fig 5E). In addition we demonstrate using surface protein biotinylation that that same amount of Plexin-A4 is present on the cell surface (Fig 5F).

3) The last paragraph of Results sustains an important conclusion (“the cis interaction between Sema6A and Plexin-A4 requires different domains that those utilized for ligand binding in vivo”), which is weakly supported by experimental data. In fact the data shown in Figure 6 are partly confusing when compared to their description in the text, they lack some appropriate controls, and even the labelling in the figure seems to require revision.

For example, labelling of panel C suggests that IPs were done with anti-Sema6A
antibodies, and immunoblotted with anti-myc antibody, which would detect the same protein (since Sema6A is myc-tagged) and not the associated Plexin-A4 (which in other figures is reported to be Flag-tagged).

In this set of experiments we used N-terminus myc tagged Plexin-A4 and a nontagged version Sema6A. This version of Sema6A is not detected by the anti-myc (9E10) antibodies. The IP was done with anti-Sema6A antibodies and Plexin-A4 was detected with anti-myc. We have now clarified this issue in the text: page 9 and legend to Fig 6.

Moreover, the manuscript text states that "cis binding was not abolished by deletion of either the Sema domain or the Ig domain of Plexin-A4 (Fig. 6B)"; however none of the panels in Fig.6 contains these data. Results shown in panel C may rather suggest that the delta-sema mutant of Plexin-A4 has lost the ability to associate with Sema6A while the delta-Ig mutant is proficient in this (thereby different from that stated in the text). Notably, a control experiment by co-expressing Sema6A and full-size Plexin-A4 should necessarily be included here for comparison. Moreover, Fig. 6B shows that all Plexin-A4 extracellular domain mutants are unable to bind secreted Sema6A, but there is no evidence about the expression of these mutated receptors on the cell surface (which could be jeopardized by large protein truncations). In principle, if Plexin-A4 delta-sema mutant would not be expressed on the cell surface, it could not be able to bind any ligand in trans, but still associate in cis intracellularly with Sema6A co-expressed in the same cells. Unless all these confusing points are cleared out, it is impossible to draw compelling conclusions about this important structural and molecular aspect of the study.

We now also provide the CO-IP experiment with full-size myc tagged Plexin-A4 in addition to the Plexin-A4 mutants (Fig 6D). In addition we show using cell surface immunostaining and cell surface biotinylation of the delta-Sema and delta-Ig that they are present on the cell surface (Fig-6A and Supplementary Fig 3), suggesting that the lack of binding is not due to lack cell surface expression.

As for interpretation of the Plexin-A4 mutants analysis:
We do agree with the reviewer that the major domain of interaction of Sema6A and Plexin-A4 is the Sema domain as its deletion reduces the cis association (by CO-IP) and abolishes the binding. The results with the delta-Ig argue that without it the Sema domain is not presented well for trans binding but it is accessible for cis binding, suggesting there is differential recognition of the Sema domain in cis versus trans. Interestingly, such a model was recently put forward for for Notch and Delta based on structural analysis (Cordle et al., 2008). Moreover, the clear association of Sema6A and the delta-N-terminus mutant argues that there is some level of interaction on the juxtamembrane domain.

In addition, the fact that we were not able to compete out the Sema6A-Plexin-A4 cis interaction by high levels of exogenous Sema6A (Fig 5G) supports the model that Sema6A is forming a structurally different complexes in cis versus trans.
We have modified the text for clarity to this re-phrasing the results, pages 9-10 and in the discussion page 11-12

Additional and minor points:

4) In the Discussion, the Authors raise a very intriguing issue that would deserve some analysis in the present work: is Sema3A signalling mediated by Plexin-A4-NP1 complexes affected by the co-expression of Sema6A in the cells?

We were not been able to generate reliable conditions in triple transfected cells in which NP1 and Plexin-A4 are expressed well enough to transmit a signal on the background of Sema6A expression. Previous studies have not revealed any differential response to Sema3A between DRG neurons (that express both Sema6A and Sema6B) and sympathetic neurons (that do not express Sema6A and sema6B) (Suto et al., 2005; Xu et al., 2000). Our analysis of co-expression of the constitutively active Plexin-A4 and Sema6A suggests that signaling (at least in COS cells) of
Plexin-A4 can be transmitted in the presence of Sema6A (supplementary Fig 4). Thus, it would appear that the NP1/Plexin-A4 complex functions regardless of Sema6A coexpression. We have now extended the discussion on this issue: page 12.

5) Pages should be numbered to facilitate reviewing work.

Pages are now numbered.

Referee #3

This paper represents an important advance in the study of the functions and interactions of transmembrane semaphorins and plexins. These molecules have been shown through mainly genetic analyses to be involved in many important axon guidance and cell migration decisions in the developing mammalian nervous system. Phenotypic analyses across several mutants have shown however that the phenotypic congruence across mutants that would be expected of a simple ligand-receptor interaction is not present. There are in fact highly complex and context-dependent genetic interactions between mutations in Sema6A, Sema6B, PlxnA2 and PlxnA4. The nature of their biochemical interactions, which may underlie this complexity, has to date been studied only at a fairly superficial level.

The authors present the first detailed study of the biochemical interactions between two of these molecules, Sema6A and PlxnA4. It reveals the very interesting and important finding that the co-expression in cis of Sema6A in responding cells blocks the response of PlxnA4 to Sema6A in trans. Given that many of the responding populations of cells in vivo co-express some level of Sema6A, this suggests that this modulatory mechanism is highly relevant in many contexts. The fact that the cis interaction seems to involve distinct domains from the trans interaction also has implications for the molecular logic of the system.

The experiments are very convincingly done and the level of quality very high.

I have one suggestion for an additional experiment that might add to the conclusions. Given that PlxnA4 is a receptor for Sema6B as well it would be very interesting to know whether expression of Sema6A in cis blocks the binding of Sema6B in trans (and vice versa). If this is so then it means that the implications of the interaction in cis are broader and extend beyond the functions of just the two molecules involved. It also means that some of the phenotypes observed when Sema6A is deleted may be due to heightened responsiveness of PlxnA4 to Sema6B signals, which would still be present in this background. These data would thus be highly relevant to the interpretation of the phenotypes seen (and not seen) in the single mutants and also of the epistatic interactions that have been observed in various double mutants in different parts of the brain. Addressing this point could add substantially to the impact of the paper.

We have now extended some of the results to Sema6B. Unfortunately, we were not able to obtain a functional Sema6B-Fc soluble protein, so we could not test this point by binding. Instead, we cloned the full length Sema6B as a myc tagged protein and demonstrate (with the appropriate controls) that it is able to prevent the binding of Sema6A-Fc to Plexin-A4 in trans (Supplementary Fig 2). We also refer to that in the discussion part in accordance to the reviewer comment page 13.

REFERENCES


Thank you for submitting your revised manuscript to the EMBO Journal. Your manuscript has now been seen by two of the original referees (#2 and 3) and their comments to the authors are provided below. Both referees appreciate the changes made and support publication in the EMBO Journal. Referee #2 has no further comments to the authors. I am therefore very pleased to proceed with the acceptance of the paper for publication here. Before doing so, referee #3 suggests a few minor text changes. I will leave it up to you if you want to incorporate these changes. If so then you can send me an amended word file by email along with a point-by-point response.

Once we have this sorted out then we will accept the paper.

Best wishes

Editor
The EMBO Journal

REFEREE REPORT

Referee #3

The authors have adequately addressed all my concerns, and, to my point of view, those of the other reviewers and the manuscript is substantially improved.

There are a few minor points that might be amended:

Page 6: it states that Plexin-A2 can serve as a receptor for Sema6A in hippocampal neurons. This has not been shown. What has been shown is that co-expression of PlxnA2 in the same cells as Sema6A blocks the response of PlxnA4-expressing mossy fibres to the Sema6A. PlxnA2 has been shown, based on genetic evidence, to act as a receptor for Sema6A in cerebellar granule cells and in spinal cord motor neurons.

Page 13: This is also not described quite accurately - at least, PlxnA2 is not acting functionally as a Sema6A receptor in this context. This should be stated more clearly. (Though the conclusion is correct that co-expression may block ligand function of Sema6A). There is also evidence from Renaud et al that co-expression of Sema6A and PlxnA2 in cerebellar granule cells may modify activity of PlxnA2 cell-
autonomously (presumably through an interaction in cis). This was speculated on in that paper and might bear comment in this discussion.

2nd Revision - authors' response  
10 June 2010

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We agree with the reviewer that Plexin-A2 was not shown to serve as a signaling receptor for Sema6A in the hippocampus. Indeed the best demonstration that Plexin-A2 can mediate Sema6A signaling comes from genetic studies of cerebellar granule cells spinal cord motor neurons. The text in p6 has been revised accordingly and additional reference was added.

Page 13: This is also not described quite accurately - at least, PlxnA2 is not acting functionally as a Sema6A receptor in this context. This should be stated more clearly. (Though the conclusion is correct that co-expression may block ligand function of Sema6A). There is also evidence from Renaud et al that co-expression of Sema6A and PlxnA2 in cerebellar granule cells may modify activity of PlxnA2 cell-autonomously (presumably through an interaction in cis). This was speculated on in that paper and might bear comment in this discussion.

Here we also agree with the reviewer’s comment and we have changed the text accordingly. We also incorporated another comment in the discussion regarding the role of Plexin-A2 cis inhibition by Sema6A in cerebellar granule cells.