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Insights into congenital stationary night blindness based on the structure of G90D rhodopsin

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(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 14 February 2013

Thank you for the submission of your manuscript to EMBO reports. It was sent to three referees and so far we have received reports from two of them, which are copied below. As both referees feel that the manuscript is interesting and recommend that you should be given a chance to revise it, I would like to ask you to begin revising your manuscript according to the referees' comments. Please note that this decision is made in the interest of time, and that it is subject to change should the third referee offer very strong and convincing reasons for this.

As you will see, referee 1 points out that the conclusion that G90D opsin shows biased signaling towards transducin needs to be toned down. In addition, both referees have (minor) technical comments and ask for clarifications.

Given these positive referee comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in

the next, final version of the manuscript.

The revised manuscript may not exceed 30,000 characters (including spaces and references) and 5 figures plus 5 supplementary figures, which should directly relate to their corresponding main figure. The current character count largely exceeds our limits, and the manuscript text therefore needs to be shortened. Shortening may be made easier by combining the Results and Discussion section, which may eliminate some redundancy that is inevitable when discussing the same experiments twice. Parts of the materials and methods can further be moved to the Supplementary Information, however, please note that the materials and methods essential for the understanding of the experiments described in the main manuscript file must remain in the main text.

We also recently decided to offer the authors the possibility to submit "source data" with their revised manuscript that will be published in a separate supplemental file online along with the accepted manuscript. If you would like to use this opportunity, please submit the source data (for example entire gels or blots, data points of graphs, additional images, etc.) of your key experiments together with the revised manuscript.

We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

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I look forward to seeing a revised version of your manuscript as soon as it is ready.

REFEREE REPORTS:

Referee #1:

The manuscript describes three structures of the G90D rhodopsin mutant causing CSNB. Two structures (G90D-GalphaCT and G90D rhodopsins) have decent resolution, whereas the G90D opsin structure was solved at a lower resolution. All three structures represent G90D in the active state. Dark-state G90D crystals could not be obtained. The structures show convincingly how the aspartate at position 90 interacts with K296 interfering with the E113-K296 activation switch and the binding of retinal. The comparison of the G90D structures to models with the other three residue substitutions causing CSNB is thoughtful. The manuscript also provides data on the thermal stability and data on phosphorylation by GRK1 and arrestin 1 binding to wild-type rhodopsin and G90D embedded into nanodiscs. The discussion is well founded to relate the crystal structures to suggestions that have been proposed in literature to explain the increased basal activity of G90D. The conclusion, that G90D shows biased signaling towards transducin is somewhat overstated, see below.

1: As explained late in the text on page 9, the basal activity of only a few G90D molecules produces a maintained activation leading to night blindness in mice. The authors have undoubtedly solved the structures of active-state G90D rhodopsin. However, it would be beneficial for the non-rhodopsin scientists to clarify early in the manuscript that the active G90D rhodopsin is not the predominant conformation in vivo. Currently, the manuscript leaves such an impression until one arrives at page 9.

2: The authors are very assertive in the description of the G90D mutant being biased towards transducin signaling. In the arrestin binding experiments, only the P-opsin form of G90D shows a

significant reduction in arrestin binding; in contrast, the P-G90D-rho* form shows only a small reduction in arrestin binding compared to wild-type rhodopsin. In normal vision, the major transducin-activating form is the meta-II rhodopsin conformation, whereas the opsin* form activates transducin much less efficiently. It may not be known in CSNB mice or patients what the proportion of G90D with retinal in its binding pocket is compared to G90D opsin; thus the species with the major contribution towards signaling in vivo seems uncertain. Biased signaling depends ultimately on the exact conformation of the intracellular surface of the GPCR. The structure of G90D opsin was solved at low resolution (3.9 angstrom) preventing a detailed comparison of its intracellular surface to that of the G90D-GalphaCT and G90D rhodopsin structures. The former species shows reduced arrestin binding, the latter much less so. In addition, arrestin binding is reduced but not abolished, and the authors do not present data of transducin activation to support their claim. The experimental observations in Fig. 6 are certainly interesting, but the assertive statement that the G90D opsin is biased towards transducin would need to be put into perspective.

3: page 5 line 8: Please provide a reference for the statement "constitutive activity typically correlates with lower stability due to increased flexibility of the protein".

4: page 5 line 13: The authors state that they performed refinement with different retinal isomers and concluded that the electron density was attributed to a mixture of cis retinal isomers. Did the authors compare the b-values obtained from the different refinement exercises to obtain an estimate whether there is a predominant form of retinal present in the mixture of species, or whether this is a mixture without preference for a particular isomer? This information may or may not be obtainable at the given data quality of the retinal, but if so, could be added into the Fig. 4 legend (rather than commenting in the text) as it would be a mechanistic detail.

5: page 5 line 14 and Fig. 4: Please clarify which structure was used in Fig. 4: G90D-GalphaCT (light-activated rhodopsin + GalphaCT) or G90D (light-activated rhodopsin).

6: It appears that the electron density for the K296 side chain is weaker than electron density for other residues shown in Fig. 4. Please provide data on the flexibility of K296 in view that the K296 side chain makes a salt bridge to D90 and thus should be highly ordered.

7: Table S1: Missing from Table S1 are Ramachandran values for protein favored and allowed regions and outliers; the b-factor needs "(Å2)" (angstrom^2) annotation. Please provide highest resolution shell data for number of reflections of G90D opsin.

8: Table S1: The G90D structure lists under b-factor the value of 76.9 for the solvent. The resolution for the G90D structure is 3.3 angstrom. Are the water molecules observed highly ordered water molecules, also seen in the G90D-GalphaCT structure; or if not, can one actually see water molecules in the G90D structure? Please clarify/comment.

Referee #2:

The manuscript "Insights into congenital stationary night blindness based on the structure of active G90D rhodopsin" by Singhal et al describes a structural and biochemical study to understand the molecular mechanisms leading to Congenital Stationary Night Blindness (CSNB). It is a very carefully conducted and comprehensive study that starts with the crystal structures of several forms of the G90D rhodopsin variant and ends with an insightfull discussion of the structural and biochemical data in the context of the pathological situation of CSNB and possible mechanisms.
G90 is a position in the rhodopsin amino acid sequence that when substituted with aspartic acid yields a rhodopsin variant that causes CSNB while when substituted with valine yields a rhodopsin variant that causes retinitis pigmentosas (RP).
The findings in the paper point to a structural origin for clustering of CNSB causing rhodopsin mutants around the retinal binding pocket. The common structural theme of these CNSB causing mutants is the formation of specific additional interactions involving K296 in the active state. This is in line with good folding properties of CSNB mutants in contrast with RP mutants. The results provided in the paper thus underline the hypothesis that both diseases (RP and CNSB) are likely caused by different mechanism, although they are similar in their symptoms.
Further biochemical investigations of the G90D variant point to a signalling bias that favors Gt

activation over arrestin-1 binding and receptor desensitization.

Minor remarks:

1) p 7: Which other RP mutants were included in the modelling? Please include this information.
2) p.8: The reference to figure 2 in the context of arrestin binding is wrong, please change to Fig. 6 b-d.
3) When describing the differences of arrestin binding to P-Opsin G90D in comparison to wt it would be nice to clearly state what are differences.
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1st Revision - authors' response 12 March 2013

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The manuscript also provides data on the thermal stability and data on phosphorylation by GRK1 and arrestin 1 binding to wild-type rhodopsin and G90D embedded into nanodiscs. The discussion is well founded to relate the crystal structures to suggestions that have been proposed in literature to explain the increased basal activity of G90D.

We thank referee#1 for his interest and the recommendation to publish our manuscript. The remaining concerns and suggestions on how to improve the manuscript are addressed below.

1: As explained late in the text on page 9, the basal activity of only a few G90D molecules produces a maintained activation leading to night blindness in mice. The authors have undoubtedly solved the structures of active-state G90D rhodopsin. However, it would be beneficial for the nonrhodopsin scientists to clarify early in the manuscript that the active G90D rhodopsin is not the predominant conformation in vivo. Currently, the manuscript leaves such an impression until one arrives at page 9.

The predominant *in vivo* conformation of rhodopsin entirely depends on the predominant conditions, i.e. whether the visual system is dark-adapted or continuously stimulated during daylight. We state in the third and fourth sentence of the results and discussion section that we solved active state G90D structures and that no crystals of the rhodopsin dark state could be grown.
We presented a necessarily short introduction to the rhodopsin photocycle, providing context for non-rhodopsin scientists. The relevance of our findings to CSNB is discussed at the end of the discussion section when all results are brought into context.

The conclusion, that G90D shows biased signaling towards transducin is somewhat overstated, see below.

2: The authors are very assertive in the description of the G90D mutant being biased towards transducin signaling. In the arrestin binding experiments, only the P-opsin form of G90D shows a significant reduction in arrestin binding; in contrast, the P-G90D-rho form shows only a small reduction in arrestin binding compared to wild-type rhodopsin. In normal vision, the major transducin-activating form is the meta-II rhodopsin conformation, whereas the opsin* form*

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We thank the reviewer for these insightful comments. Several groups have previously reported increased constitutive activity of G90D opsin towards transducin (Gt). Therefore, we did not see the need to confirm this well-established fact in our work. Indeed for many years this increased activity was believed to be the predominant cause of CSNB. In this respect we think it very relevant that G90D opsin binds the deactivating protein arrestin less efficiently. It further makes sense to attribute this reduction to a reduced conformational flexibility resulting from the identified G90DK296 salt bridge that links TM2 and TM7. The conformational dynamics of TM7/H8 have been implicated in binding of arrestin [1]. Several recent publications on related beta-adrenergic receptors show the importance of TM2 and TM7 in binding of arrestin biased agonists [2,3]. We therefore believe this example of a pathological mutation that has opposite effects on Gt and arrestin binding will be of great interest for the wider GPCR community. However, in line with the referee's suggestion we reduced and toned down the discussion of G90D bias.

We agree with the referee that further experiments (including further *in vivo* experiments to establish the species with major contribution to signaling in G90D patients and mice) are needed to quantify the biasing effect on different rhodopsin forms and clarify the impact on CSNB. We therefore merged the two sections "The G90D mutation reduces binding of arrestin" and "Relevance to GPCR biased signaling" and significantly shortened the discussion of biased signaling. The resulting section is more carefully phrased and does not overstate our data.

3: page 5 line 8: Please provide a reference for the statement "constitutive activity typically correlates with lower stability due to increased flexibility of the protein".

Although published data support this statement, we had to remove it to make the manuscript more concise.

4: page 5 line 13: The authors state that they performed refinement with different retinal isomers and concluded that the electron density was attributed to a mixture of cis retinal isomers. Did the authors compare the b-values obtained from the different refinement exercises to obtain an estimate whether there is a predominant form of retinal present in the mixture of species, or whether this is a mixture without preference for a particular isomer? This information may or may not be obtainable at the given data quality of the retinal, but if so, could be added into the Fig. 4 legend (rather than commenting in the text) as it would be a mechanistic detail.

We extensively tried to identify the predominant retinal isomer in our G90D structures by comparing density correlation coefficients and b-factors from crystallographic refinements. Based on this analysis the remaining retinal is clearly a mixture of cis retinals without one predominant isomer. In Fig. 4 we indicate that 9-13-di-cis fits the difference density to some extent. Indeed thermal isomerization of free 11-cis and all-trans retinal leads to the formation of mixtures containing 9-cis, 13-cis and 9-13-di-cis retinal [4]. However, as crystallographic refinement with 9-13-di-cis did not lead to a satisfactory solution, we decided to not overstate this point and leave the question of the predominant retinal isomer open.

5: page 5 line 14 and Fig. 4: Please clarify which structure was used in Fig. 4: G90D-GalphaCT (light-activated rhodopsin + GalphaCT) or G90D (light-activated rhodopsin).

We thank the reviewer for this suggestion. We have used the G90D-GaCT data to prepare this figure, as it has the highest resolution. We have clarified this in the Figure legend.

6: It appears that the electron density for the K296 side chain is weaker than electron density for other residues shown in Fig. 4. Please provide data on the flexibility of K296 in view that the K296 side chain makes a salt bridge to D90 and thus should be highly ordered.

Part of the weaker appearance of the K296 side chain in Figure 4 is due to the angle in which the side chain is shown. The G90D side chain has been omitted during the refinement used for this Figure to illustrate the presence of the mutation. This omission also affected the density of the interacting K296 side chain. When both G90D and K296 are omitted a clear continuous difference density appears demonstrating the presence of the linking salt bridge. Our final maps calculated with both G90D and K296 show strong density for the K296 side chain, which is continuous up to a sigma level of 3.0. A further indication for a low flexibility of the K296 side chain are its low b factors, which are 58 Å2, close to the values of 50-55 Å2 for the well ordered protein backbone in other parts of the ligand binding pocket. Together the low b factors and the strong density leave not doubt that the salt bridge is highly ordered.

7: Table S1: Missing from Table S1 are Ramachandran values for protein favored and allowed regions and outliers; the b-factor needs "(Å2)" (angstrom^2) annotation. Please provide highest resolution shell data for number of reflections of G90D opsin.

We thank the reviewer for this suggestion. We have added the requested values to table S1.

8: Table S1: The G90D structure lists under b-factor the value of 76.9 for the solvent. The resolution for the G90D structure is 3.3 angstrom. Are the water molecules observed highly ordered water molecules, also seen in the G90D-GalphaCT structure; or if not, can one actually see water molecules in the G90D structure? Please clarify/comment.

We agree with the referee that waters are very difficult to spot at the 3.3 Å resolution of the G90D structure and would not have included them if this were the only data available. However many waters can be clearly identified in the 2.9 Å G90D-GaCT structure and are also present in other higher resolution metarhodopsin-II structures [5]. We therefore decided to include waters in cases where difference density was found at known water positions and where inclusion of waters improved the G90D model.

Referee #2:

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We thank referee #2 for this assessment and the interest in our work.

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The findings in the paper point to a structural origin for clustering of CNSB causing rhodopsin mutants around the retinal binding pocket. The common structural theme of these CNSB causing mutants is the formation of specific additional interactions involving K296 in the active state. This is in line with good folding properties of CSNB mutants in contrast with RP mutants. The results provided in the paper thus underline the hypothesis that both diseases (RP and CNSB) are

likely caused by different mechanism, although they are similar in their symptoms.
Further biochemical investigations of the G90D variant point to a signalling bias that favors Gt activation over arrestin-1 binding and receptor desensitization.

We thank the reviewer for pointing out that the comparison of CSNB and RP mutants is a very interesting aspect of our work.

Minor remarks:

1) p 7: Which other RP mutants were included in the modelling? Please include this information.
All RP mutations included in this comparison are shown in the corresponding Figure 4.

2) p.8: The reference to figure 2 in the context of arrestin binding is wrong, please change to Fig. 6
b-d.

We thank the referee for spotting this mistake. It has been corrected in the resubmitted version of the manuscript.

3) When describing the differences of arrestin binding to P-Opsin G90D in comparison to wt it would be nice to clearly state what are differences.

Binding of P-Opsin G90D to arrestin was reduced by 70% compared to wild type P-Opsin. We included this information in the revised version of the manuscript.

4) p. 10: The reference to Fig. 2 in the context of the models causing CSNB is wrong, please change
to Fig. 7 .

We have corrected the corresponding Figure reference.

References:

1. Kirchberg K, Kim T-Y, Möller M, Skegro D, Dasara Raju G, Granzin J, et al. Conformational dynamics of helix 8 in the GPCR rhodopsin controls arrestin activation in the desensitization process. Proc. Natl. Acad. Sci. U.S.A. 2011 Nov 15;108(46):18690–5.

2. Warne T, Edwards PC, Leslie AGW, Tate CG. Crystal Structures of a Stabilized β(1)-Adrenoceptor Bound to the Biased Agonists Bucindolol and Carvedilol. Structure. 2012 May 9;20(5):841–9.

3. Liu JJ, Horst R, Katritch V, Stevens RC, Wüthrich K. Biased signaling pathways in β2-adrenergic receptor characterized by 19F-NMR. Science. 2012 Mar 2;335(6072):1106–10.

4. Groenendijk GW, Jacobs CW, Bonting SL, Daemen FJ. Dark isomerization of retinals in the presence of phosphatidylethanolamine. Eur. J. Biochem. 1980 May;106(1):119–28.

5. Choe H-W, Kim YJ, Park JH, Morizumi T, Pai EF, Krauss N, et al. Crystal structure of metarhodopsin II. Nature. 2011 Mar 31;471(7340):651–5.

2nd Editorial Decision 17 March 2013

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

There are only two minor issues that still need to be fixed. Please specify the error bars and number of experiments (n) in the legends for supplementary figures 2 and 3. Can you please send us a new supplementary material file that has this information? You can send it as email attachment and reply to this email.
Please also include the pdb codes for your structures in the main materials and methods section. If you agree, we can copy the sentence from the supplementary materials section to the main materials and methods. Please let us know if this is OK.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

REFEREE REPORT:

Referee #1
The authors have adequately dealt with the reviewers' comments. The manuscript describes structures of the G90D rhodopsin explaining convincingly how the aspartate at position 90 interferes with retinal binding and normal rhodopsin function.

