Structural role of the T94I rhodopsin mutation in congenital stationary night blindness

Ankita Singahl, Ying Guo, Milos Matkovic, Gebhard Schertler, Xavier Deupi, Elsa Yan, and Jörg Standfuss

Corresponding author: Jörg Standfuss, Paul Scherrer Institut

Review timeline:

<table>
<thead>
<tr>
<th>Event</th>
<th>Date</th>
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<tbody>
<tr>
<td>Submission date</td>
<td>04 May 2016</td>
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<tr>
<td>Editorial Decision</td>
<td>27 June 2016</td>
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<tr>
<td>Revision received</td>
<td>05 July 2016</td>
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<tr>
<td>Accepted</td>
<td>07 July 2016</td>
</tr>
</tbody>
</table>

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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.)

Thank you for the submission of your research manuscript to our journal. I am very sorry for the unusual delay in getting back to you, we have only now received the full set of referee reports that is copied below.

As you will see, all referees acknowledge that the findings are interesting, and they have only a few suggestions for how the study could be improved. Given the small number of concerns and their nature, I think that all of them should be addressed.

We would thus 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. Please address all referee concerns in a complete point-by-point response. 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.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Given the 4 main figures, I suggest that we publish your study as a short report. For short reports, the revised manuscript should not exceed 25,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must be combined, and the entire materials and methods must be included in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were
performed, and the bars and error bars (e.g. SEM, SD) for supplementary figure 2. Please note that supplementary figures and tables are called Expanded View at EMBO press now. These data are integrated in the manuscript text online and expand when clicked.

We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS

Referee #1:

Singhal et al. present a multidisciplinary analysis of the mutant rhodopsin T94I, which is associated with congenital stationary night blindness (CSNB). CSNB is an exceedingly interesting visual disorder characterized by a loss of scotopic vision due to a change in rod cell visual threshold, which physiologically is caused by so-called "dark noise." There are several rhodopsin mutations associated with CSNB, most notably G90D. In fact, the current paper is really a "compare and contrast" study of T94I and G90D. The main contribution here is the report of a crystal structure for T94I, which turns out to be highly interesting and informative. However, since only the "active state" structure of T94I (and G90D, for that matter) have been solved, there are some significant holes in our understanding of the underlying molecular pathophysiology when analyzing the crystal structure alone. To fill these holes, the authors carry out very nice thermal stability assays that include retinal chromophore extractions and HPLC analysis. In addition, nice MD simulations are carried out using rhodopsin as a comparator. The overall conclusion is that T94I causes effects at the Schiff base (SB) environment as expected, but the effects are not the same as with G90D. In fact, it is the SB hydrolysis rate that is most affected by T94I so that the active MII-like state is prolonged. How this occurs - through a loss of an active site water and steric affects on the SB reaction geometry - is interesting and nicely discussed. Finally, the concluding section presents a nice discussion of what is known about the biochemistry and spectroscopy of CSNB mutants. The experiments presented are technically satisfactory and employs established methodologies from collaborative groups with a long history of similar work. The only weakness of the work is that some of the conclusions have to be a bit speculative because of the lack of a crystal structure of the dark state. I have only a very few minor issues for the authors to consider:

1. The abstract could be tightened up a bit. For example, the main conclusion is not clearly stated because the prolongation of MII is introduced, and then there is a comparison with G90D and some comment about the "E113 SB activation switch." What is the meaning of "activation switch?" I understand the most visual pigment aficionados will know what that means, but the term should be used only after some explanation in the main text. Something like "changes the electrostatic environment of the SB in the dark state" would be maybe better for the abstract.

2. The term "rhodopsin" almost always will imply the presence of the 11-cis-retinal chromophore, so the entire paper should be edited for clarity and the term opsin should be used more generously where needed.

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4. The form of the T94I mutant crystalized should be stated more clearly even in the introduction. It was not until the second or third paragraph of the results that I realized what the crystal structure was. Until then, I was hoping that it would be the "dark" state, so it was a bit of a let down.
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This paper by Singhal et al is well written, concise and easy to read, and very appropriate for EMBO reports. The authors determined the structure of the congenital stationary night-blindness mutant (CNSB), T94I, in the active, MII state to 2.3 Å resolution, then compared it to that of another CNSB mutant, G90D, to try and glean mechanistic insights into the underlying cause of this disease.

Interestingly, even though both mutants lead to the same disease phenotype (CNSB), the structure of the two mutants were significantly different around the retinal Schiff-base attachment site in the active state.

To better understand this somewhat paradoxical result - same disease phenotype, different underlying molecular properties for the receptor involved - the authors considered other factors that could be the underlying result in CNSB.

Since their structure was of the active state, not the inactive, (dark) receptor state, the authors then carried out extensive biochemical studies of receptor stability and retinal configuration present for both mutants after thermal perturbation.

The results clearly show that both mutants are less thermally stable than wild-type rhodopsin, with the T94I showing the least stability. They also carried out molecular dynamic simulations of both mutants in the dark state, to assess if significant differences in the dark-state structure of both mutants could lead to the disease phenotype.

Based on these studies, the authors propose that the G90D and T94I mutations likely result in CNSB because they enable the receptor to transiently adopt an active conformation in the dark state, resulting in low-level signaling activity, even when bound by the inverse agonist, 11-cis retinal.

Overall, I think this is an important, carefully excerpted (and reasoned) piece of work, and thus is very appropriate for publication in a high-impact journal like EMBO Reports. The topic is timely, the experiments were carefully executed, and the analysis very well reasoned.

I have only a few minor issues, related to the text.

There are some typos throughout parts of the manuscript. For example, on page 3, "the same disease phenotype than...", and later, in the Results and Discussion "In a first step...".

Also, in Figure 3, the authors should define what "c-c" means. In that figure, it also looks like the polyene chain of the retinal has rotated slightly, with a a shift in the location of the ionone-ring for the MD simulation of the T94I protonated E113 structure. The author might want to discuss if this is indeed occurring, and if so, how that might help lead to the active receptor formation.

Finally, the last sentence of the abstract should start with "We propose...", since this work has not formally shown that the mutations cause an increase in dark-state basal signaling, and thus the resulting background noise would lead to CSNB (although I agree with the analysis, this conclusion can only be inferred based on the data presented here.

In summary, I think this is a very good paper, well written, and should be published in EMBO Reports.

Referee #3:

The manuscript describes the molecular mechanisms underlying a mutation that leads to hereditary night blindness. The authors show that the mutation results in destabilization of the basal state of the receptor, which is proposed to decrease the signal to noise ratio in the visual system and thereby cause visual impairment.

Overall the manuscript is well written and clear. The combination of crystallography,
biochemical/biophysical analysis and molecular dynamics simulations provides solid support for the key conclusions, and the experiments are technically sound. Crystallographic data are solid and sufficient to support the conclusions. There are a few minor points for correction detailed below, but overall the manuscript is excellent and I recommend publication without reservation.

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CE and NZ should read Cε and Nζ

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There is a mismatch of the text with the figures. The panels 2E-H are describing the SB hydrolysis (that in the text is labeled as 2 I-L) and vice versa with the thermal isomerization.

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Supplementary Figure 1:
Line 6, "and" should be "an".

Supplementary Table 1:
"a, b, g" should read "α, β, γ" (i.e., alpha, beta, gamma).

Thank you for considering our Manuscript “Structural role of the T94I rhodopsin mutation in congenital stationary night blindness”. We were delighted by the positive comments of the three referees and have integrated their remaining suggestions into a revised version of the manuscript. The new version fully complies with the EMBO Reports format and we hope you find it suitable for publication.

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We thank the referee for the summary and the positive assessment of our work. We will address all remaining issues and recommendations below.

- The abstract could be tightened up a bit. For example, the main conclusion is not clearly stated because the prolongation of MII is introduced, and then there is a comparison with G90D and some comment about the "E113 SB activation switch." What is the meaning of "activation switch?" I understand the most visual pigment aficionados will know what that means, but the term should be used only after some explanation in the main text. Something like "changes the electrostatic environment of the SB in the dark state" would be maybe better for the abstract.

We see the point of the referee that the E113 SB activation switch first needs to be introduced, for which there is no space in the abstract. We therefore changed the sentence to "... and the hydrophobic T94I2.61 mutation alter the dark state by weakening the interaction between the Schiff base (SB) and its counterion E1133.28." That this interaction is part of a critical activation switch is introduced later in the manuscript. We further clearly state now in the abstract that the structure of T94I represents the active state.

- 2. The term "rhodopsin" almost always will imply the presence of the 11-cis-retinal chromophore, so the entire paper should be edited for clarity and the term opsin should be used more generously where needed.

Yes the terminology of rhodopsin is unfortunately not always clear and some use rhodopsin only when the 11-cis retinal is present. Throughout the manuscript we use rhodopsin for the light-receptor itself and specify to dark state when 11-cis retinal is present, opsin in absence of ligand, metarhodopsin-II for activated receptor with covalently bound all-trans retinal, or active conformation in cases when all-trans is not covalently bound but the protein in the active conformation. We think this terminology is clear (in agreement with the other referees) and maintain it throughout the revised version of the manuscript.

- 3. P.3, same AS, not same THAN

The typo has been corrected. We thank the referee for spotting it.

- 4. The form of the T94I mutant crystalized should be stated more clearly even in the introduction. It was not until the second or third paragraph of the results that I realized what the crystal structure was. Until then, I was hoping that it would be the "dark" state, so it was a bit of a let down.

To address the concern we now clarify the conformation in the abstract as follows “Here, we present the light activated conformation of CSNB causing T94I2.61 rhodopsin at 2.3 Å resolution.” We also would like to note that already the initial manuscript clarified the state at the end of the introduction as follows “In order to elucidate this cause, we have solved the crystal structure of the T94I2.61 mutant in the metarhodopsin-II active conformation.” We had no intention to hide this fact and think our biochemical analysis provides clear evidence why the fragile CSNB dark state did not crystallize.

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- This paper by Singhal et is well written, concise and easy to read, and very appropriate for EMBO reports. The authors determined the structure of the congenital stationary night-blindness mutant (CNSB), T94I, in the active, MII state to 2.3 Å resolution, then compared it that of another CNSB mutant, G90D, to try and glean mechanistic insights into the underlying cause of this disease.
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I have only a few minor issues, related to the text.

- There are some typos throughout parts of the manuscript. For example, on page 3, "the same disease phenotype than...", and later, in the Results and Discussion "In a first step...". The typos have been corrected.

- Also, in Figure 3, the authors should define what "c-c" means.

The depiction “c-c” stands for the stabilizing disulfide bridge described in reference 18 (Xie et al., 2003.). It is introduced in the main text but to further clarify its use in the figures we have added a description to the caption of figure 1.

- In that figure, it also looks like the polyene chain of the retinal has rotated slightly, with a shift in the location of the ionone-ring for the MD simulation of the T94I protonated E113 structure. The author might want to discuss if this is indeed occurring, and if so, how that might help lead to the activate receptor formation. Indeed the rotation the beta-ionone ring with respect to the polyene chain changed within all simulations and not only for T94I. We attributed this to some degree of positional freedom within the hydrophobic binding pocket but as it was not specific to the disease mutants we did not further discuss it. The rotation may also just be due to inaccuracy of the MD simulations as all crystal structures indicate a fixed position of both polyene and β-ionone ring. As a further clarification we have added the following sentence to the figure caption “In contrast to the crystal structures the orientation of the retinal β-ionone ring in the simulations was variable with respect to the polyene chain. This indicates a degree of positional freedom within the hydrophobic binding pocket.”

- Finally, the last sentence of the abstract should start with "We propose..", since this work has not formally shown that the mutations cause an increase in dark-state basal signaling,
and thus the resulting background noise would lead to CSNB (although I agree with the analysis, this conclusion can only be inferred based on the data presented here.

In response to the suggestion the last sentence of the abstract now reads “We propose that this interference with the tight regulation of the dim light photoreceptor rhodopsin increases background noise in the visual system and causes the loss of night vision characteristic for CSNB patients.”

In summary, I think this is a very good paper, well written, and should be published in EMBO Reports.

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• The manuscript describes the molecular mechanisms underlying a mutation that leads to hereditary night blindness. The authors show that the mutation results in destabilization of the basal state of the receptor, which is proposed to decrease the signal to noise ration in the visual system and thereby cause visual impairment.

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We are glad the reviewer enjoyed reading our manuscript and give our thanks for the recommendation to publish without reservation.

• Page 5, line 12: CE and NZ should read Cε and Nζ

This has been corrected.

• Page 6, second paragraph: There is a mismatch of the text with the figures. The panels 2E-H are describing the SB hydrolysis (that in the text is labeled as 2 I-L) and vice versa with the thermal isomerization.

The Figure has been adapted to fit better to the flow of the main text.

• Page 8, last line: "In G90D displaces the retinal counterion..." This sentence isn't clear to me, maybe missing a word?

We changed the sentence to “G90D displaces the retinal counterion E1133 in the dark state [7] and can form a salt bridge with K296 to stabilize an active opsin conformation with mixed retinal isomers [16].” This should be clearer.

• Figure 1: The bottom panels are a bit small and the text (particularly in the upper panels) is too small to read clearly.

We have reformatted Figure 1 in the revised manuscript to increase readability. Text size has been increased to 12 points.

• Supplementary Figure 1: Line 6, "and" should be "an".

This has been corrected in the revised version of the manuscript.

• Supplementary Table 1: "a, b, g" should read "α, β, γ" (i.e., alpha, beta, gamma).

Supplementary Table 1 has been corrected.
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.
Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data. Please follow the guidelines set out in the authors’ guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (e.g. cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range.
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired or unpaired), simple p-tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P-values ≥ but not < c; definition of “center values” as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non-applicable).

B- Statistics and general methods

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

NA.

1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

NA.

1.c. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria prespecified?

NA.

1.d. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.

NA.

2. For animal studies, include a statement about randomization even if no randomization was used.

NA.

3.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes, please describe.

NA.

3.b. For animal studies, include a statement about blinding even if no blinding was done.

NA.

4. For every figure, are statistical tests justified as appropriate?

NA.

5.a. Did the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess this.

NA.

5.b. Where there an estimate of variation within each group of data?

NA.

5.c. Is the variance similar between the groups that are being statistically compared?

NA.
D- Animal Models

- Dual use research of concern
- Data Accessibility
- Animal Models

E- Human Subjects

- Identify the committee(s) approving the study protocol.
- Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.
- For publication of patient photos, include a statement confirming that consent to publish was obtained.
- Report any restrictions on the availability (any form of) of human data or samples.
- Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.
- For phase II or III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under Reporting Guidelines. Please confirm you have submitted this list.
- *For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under Reporting Guidelines. Please confirm you have followed these guidelines.

F- Data Accessibility

- Provide accession codes for deposited data. See author guidelines, under Data Deposition.
- Data deposition in a public repository is mandatory for:
  a. Primer, DNA and RNA sequences
  b. Microarray data
  c. Protein data for small molecules
  d. Functional genomic data
  e. Proteomics and molecular interactions.
- Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreements written into the study, such data should be deposited in one of the major public access controlled repositories such as BioGPS (see link list at top right) or EGA (see link list at top right).
- In lieu of possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section.

Examples:
- Primary Data
  - Reference Data
  - Huang L, Brown AF, Lei M (2012). Crystal structure of the TRIB2-domain of TET2 and the C3H of TR. Protein Data Bank 4026
  - AF-MRI analysis of human histone deacetylase interactions in C3-M T cells (2013). PRO59000020

- Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized formats (SBML, CoMFA) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit the model in a public database such as Biomodels (see link list at top right) or BioModelsDB (see link list at top right). If a compiler source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.

G- Dual use research of concern

- To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., antilipoedema (see link list at top right).
- Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for exopolysaccharide contamination.

* For all hyperlinks, please see the table at the top right of the document.