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Ultrafast optogenetic stimulation of the auditory pathway by targeting-optimized Chronos

Daniel Keppeler, Ricardo Martins Merino, David Lopez de la Morena, Burak Bali, Antoine Tarqiun Huet, Anna Gehrt, Christian Wrobel, Swati Subramanian, Tobias Dombrowski, Fred Wolf, Vladan Rankovic, Andreas Neef, Tobias Moser

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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 13th Jun 2018

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

As you can see from the comments, the referees find the analysis important and support publication here. They raise a number of constructive issues that I would like to ask you to resolve in a revised version. The concerns raised are clearly outlined below. Let me know if we need to discuss anything further.

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://emboj.embopress.org/about#Transparent_Process

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 may be able to grant an extension.

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

REFEREE REPORTS:

Referee #1:

Keppeler, Merino, de la Morena et al. improved an optogenetic tool, called Chronos, for stimulating spiral ganglion neurons in the ear towards the generation of an optogenetic optical cochlear implant. First, they overcame limited membrane localization by adding a trafficking signal (TS), and ER export signal (ES) leading to Chronos-ES/TS. Next, the authors have used an advanced AAV serotype (AAV-PHP.B) facilitating better optogene expression in spiral ganglion neurons. Both improvements resulted in an advanced functional performance, carefully tested in vitro and in vivo, overcoming the poor kinetics of previously used optogenetic tools. The experiments are well performed, underlying the authors' claims that Chronos-ES/TS is superior than unmodified Chronos or ChR2. The text is clearly written, comprehensive and not overstating the results. This work clearly demonstrates the need of optimizing optogenetic tools for specific applications. In addition, the transfer and combination of previously reported modifications (ES/TS and the AAV serotype) was key to success and can be inspiring to the entire optogenetic field: Whenever expression levels are poor, one needs to tailor the optimal optogenetic tool and AAV.

Some minor points:
• Page 7/ Figure legend 2A: Does bGH refer to the pA signal? Please clarify.
• Page 7 Figure 3: The data would be more convincing by adding a quantification?
• Page 8: Why is the data for AAV2/6 carrying Chronos-ES/TS not shown? It would be consistent to show.
• Page 9: The sentence "Expression of Chronos seemed weaker than that of Chronos-ES/TS, but much stronger than that reported above for AAV2/6 carrying Chronos." Is not very scientific. Would it be possible to underline this finding with statistics?
• Page 10/Figure legend 4F: The light grey boxes were first quite confusing that they represent the "contralateral, non-injected cochleae". It would be great to clarify this in the figure legend or figure.
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Referee #2:

Optogenetics is considered to be a tool in future forms of therapy for a multitude of human diseases. As with many other types of therapy, there are additional and multiple challenges for sensory diseases, in particular deafness. Keppeler et al attempted to overcome some of these challenges by engineering modifications to the channelrhodopsin Chronos, which enabled altered trafficking to the cell plasma membrane. Most compelling, they changed the temporal fidelity of the firing, a major impediment previously in optogenetics of inner ear. They then stimulated spiral ganglion neurons optically, following transduction using AAV. The AAV used, AAV-PHP.B, was previously set up for brain and Keppeler et al succeeded in using it for inner ear SGNs. A thorough characterization was done on the resulting cells and mice. The work is extremely promising for the implementation of optogenetics in conjunction with cochlear implant for future therapy.

Minor editing comments
1) Remove "For example", since the rest of the text pertains to SGN.
"For example, optogenetic stimulation of spiral ganglion neurons (SGNs) in the ear provides a future alternative to electrical stimulation used in cochlear implants."

2) Insert "a"
"...suffer from a disabling hearing impairment..."

3) Change colons to semicolons.
"...for the most common form; sensorineural hearing impairment..."
"However, the temporal fidelity of ChR2-mediated optogenetic control of SGN firing seemed limited; auditory brainstem response..."

4) Use less references, but also replace at least one with a reference that is more recent, to

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demonstrate that in 2018 eCI are still the optimal form of therapy.
"...achieving open speech comprehension, is considered the most successful neuroprosthesis (Wilson & Dorman, 2008; Middlebrooks et al, 2005; Zeng et al, 2008)

5) Add comma
"...activation of the auditory pathway up to the midbrain (inferior colliculus, IC), demonstrating..."

6) Remove "But"
"But although this had been successfully employed.."

7) Capitalize
advanced grant
research and innovation program
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8) More consistency between figures
For example, Figure 6 x axis labels are large font, bold and italics; does not match other figures
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Referee #3:

Keppeler et al. presented a study to optimize the expression of the opsin Chronos (a blue channelorhodopsin with the fastest kinetics among reported opsins) and enable reliable ultrafast optical stimulation of the auditory pathway.

The optimization strategy relies on two factors. First, the realization of an enhanced-trafficking version of Chronos obtained by adding sequences promoting endoplasmatic reticulum exit (Export Signal, ES) and plasma membrane trafficking (Trafficking Signal, TS) to Chronos (Chronos-ES/TS). A strategy already reported (Gradinaru et al., 2010), but never applied to Chronos. Second, the adoption of postnatal injection of AAV-PHP.B vector, a recently published AAV-serotype owing higher neural transduction.

The study is of great relevance for the auditory research and its clinical applications for rehabilitation from cochlear dysfunction as it aims to expand the applicability of optogenetic neuronal investigations for sound coding in spiral ganglion neurons (SGNs) of the ear which requires firing at hundreds of Hz with sub-ms precision.

However, a number of major issues needs to be addressed before publication.

1. Figure 1 shows kinetics properties for Chronos and ChR2 measured by patch-clamp recordings of opsin-expressing HEK-293T cells during photostimulation. Chronos has been measured both for 22{degree sign}C and 36{degree sign}C, showing an acceleration of the kinetics for higher temperatures. However, ChR2 recordings have been performed at 22{degree sign}C only. It should be considered the opportunity to perform recordings of ChR2 at 36{degree sign}C to rigorously compared Chronos and ChR2 at body temperature.

2. In Fig.2 authors present a quantification of the cellular distribution of the opsin in HEK-293T cells transfected with Chronos and Chronos-ES/TS based on the analysis of the fluorescent signal carried with confocal and STED images. The localization of the fluorescence appears more peripheral for the Chronos-ES/TS by looking at the representative images (FIG.2B) and by the analysis of the intensity profiles along lines crossing the cell membrane (Fig.2C). It is not clear and should be reported the number of line-profiles per cell and their distribution along the membranes in order to evaluate the robustness of the analysis.

3. Fig.2D highlights the superior membrane expression of Chronos-ES/TS by measuring the fluorescence ratio membrane/cytosol. In this case, it should be specified over which distances and

how far from the cell border have been measured the "membrane" and "cytosol" fluorescence. Moreover, also in this case, it needs to be discussed the robustness of the analysis in terms of number and distribution of line-profiles per cell. Furthermore, although the ratio membrane/cytosol fluorescence is higher for ES/TS-Chronos, it presents also a significant wider spread of data compared to Chronos. The reason for that is not clear and need to be discussed.

4. Although the analysis of fluorescence presented in Fig.2 showed superior localization of the fluorescence on the membrane for ES/TS-Chronos compared to Chronos, it appears also that the maximum fluorescence intensity at the membrane is slightly higher for Chronos than for ES/TS-Chronos (Fig.2C inset), which makes difficult to estimate the real superiority of ES/TS-Chronos in terms of activation efficiency. In order to fully quantify the differences of activation efficiency, patch-clamp recordings of light-evoked photocurrents in Chronos and ES/TS-Chronos cells should be conducted and correlated to the fluorescence analysis.

5. Fig.3 shows representative images of hippocampal neurons in culture expressing Chronos and Chronos-ES/TS transduced either by AAV2/6 or AAV-PHP.B. Although from those representative neurons it is observable an enhanced fluorescence localization on the membrane for Chronos-ES/TS, a statistical analysis of those results is completely missed, which makes difficult to properly quantify the improvements obtained. Again, also in this case, patch-clamp measurements of photocurrents or spike thresholds during illumination would give fundamental elements for the quantification of the entity of the improvements obtained with Chronos-ES/TS. Moreover, that would give insights on the functionality and the health of the cells.

6. Fig.4 presents a comparison between expression in SGNs via transuterine injections of AAV2/6 and via early postnatal injections of AAV-PHP.B vector. The representative confocal images (Fig.4B and Fig.4C) of immunolabeled cochlear cryosections show an improvement in the number of positive cells in the case of AAV-PHP.B for Chronos-expressing neurons. However, a statistical analysis for Chronos AA2/6 to indicate the fraction of positive cells and their expression level would be convenient for a more exhaustive evaluation of the improvements and to make consistent some semi-quantitative claims in the text, i.e. "In most of the cases the expression of Chronos was absent" (pag7 line6); "Exceptionally, we saw high expression levels (pag7 line7)"; "confocal images suggested poor plasma membrane expression e.g. compared to Catch"(pag7 line 10).

7. In Fig.4 Calretinin and Parvalbumin were used as generic markers of SGNs for Chronos AAV2/6 and Chronos-ES/TS AAV-PHP.B, respectively. As different markers can have a different labeling, it should be discussed how that choice can affect the estimation of the total number of cells and thus of the fraction of positive cells.

8. The analysis of Fig. 4E holds the same comments previously described for the corresponding graphs shown in Fig.2C and 2D. Moreover, comparing the results obtained on HEK cells and on SGNs, it appears that the ratio between membrane/cytosol fluorescence is inferior in SGNs (Fig.4E right vs Fig.3D) and the ratio of the maximum fluorescence at the membrane between Chronos and ES/TS-Chronos presented an inverted behavior for SGNs and HEK-cells (Fig.4E inset vs Fig.3C). It would be useful to comment these aspects.

9. Fig.4F and 4G analyze the transduction rate in the injected and non-injected cochleae. However, it is not clear and should be explained how the virus can spread from one cochleae to the other. Moreover, since the expression in the non-injected cochlea is not controlled, it should be explained how such an expression can be considered as a truthful terms of comparison to conclude that the injection of the virus does not cause loss of SGNs in the injected cochlea.

10. Fig.6 presents an analysis of optical auditory brainstem responses (oABR) induced in Chronos- and ES/TS-Chronos-expressing mice by illuminating with "1ms light pulses delivered at 10Hz". It is not clear and should be specified the total number of pulses or the total duration of the stimulus. Also, it should be better explained the protocol of measurements, in particular the number of trials/experiments conducted per each animal to produce graphs in Fig.6B,C,E,F,H,I.

11. Fig.6C shows oABR latencies. It is not fully clear and should be better specified how latencies have been calculated. Moreover, authors state that "oABR latency got shorter for Chronos-ES/TS and was relatively constant for Chronos". However, it seems that also Chronos latency decreases

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(although with a less steep slope than Chronos-ES/TS) by increasing the illumination intensity.

12. Fig.6E presents P1-N1 amplitude percentage for different illumination durations. Authors claim that oABRs could be elicited by light pulses as short as 100us with ES/TS-Chronos and 200us with Chronos. However, the plot seems to indicate the opposite, probably colors of the lines are inverted? Also it is not clear why, by incrementing the illumination durations, latencies increase for ES/TS-Chronos and decrease for Chronos, respectively.

13. Authors claim that "sizeable P1-N1 amplitudes can be detected up to 500Hz for Chronos and 1000Hz for Chronos-ES/". However, it is difficult to appreciate it from figure 6H because it seems that the green line corresponding to Chronos does not arrive up to 500Hz. In particular it is not clear if Chronos has been tested up to 1000Hz, as green symbols should appear even if they are equal to zero. Furthermore, error bars for Chronos are quite big and should be considered the opportunity to enlarge the statistics (n=4 for Chronos) to permit a more rigorous comparative analysis.

14. Fig.7 shows extracellular recordings from single putative SGN during optogenetic stimulation. Except for some circumstances (whose number should be reported in the text), mean spike probability appears to be nearly 0 for repetition rate above 500Hz (Fig.6E). Spike synchronicity with light pulses appears confined on average to around 300-400Hz (Fig.6D) and for all stimuli between 20-1000Hz the discharge rate was on average inferior to 100spikes/s. On the basis of these results it appears necessary to better discuss to which extent it can be assumed that high temporal fidelity of light-evoked spiking have been enabled and, eventually, to reformulate certain sentences (i.e., "recordings from single SGNs demonstrated high temporal fidelity of light-evoked spiking" (Abstract); "some putative SGNs were able to maintain moderate and to some extent synchronized spike rates even at pulse rates of 1000Hz"(Results, p.15); "using juxtacellular recordings we could demonstrate firing of single SGNs in response to trains of light pulses at hundreds of Hz with sub-ms temporal presision"(Discussion, p.20)).

15. oABRs could be detected up to 1000Hz (albeit with a very low P1-N1 amplitude), while mean spike probability of photostimulated SGNs drops below 10% for repetition rate above 300Hz (Fig.6E). It should be discussed the reasons for this difference.

Minors:
16. Please consider to have zooms for Fig.1B in order to be able to visualize the shapes of currents.
17. In Fig. 4E the Y-axis title and units are missed.
18. In Fig.4F it should be considered the opportunity to change the title of the axis as "YFP/GFP expression" appears as a ratio, e.g. "YFP or GFP expression"
19. Fig.5 presents oABRs recorded in SGNs following transuterine injections of AAV2/6. Due to the limited number of animals exhibiting oABRs in this case (3 out of 120), it should be considered if it is necessary to have it as main figure (and in this case it should be better emphasized the relevancy of this result) or as supplementary.
20. In Fig.5 it is necessary to put a temporal scale bar.
21. In Fig.6 it would be convenient to show the tick marks in the logarithmic X-scale ranges in each graph to help visualization.
22. It is necessary to insert in the methods an explanation for the calculation of the vector strength shown in Fig. 7C.
23. It is necessary to report more consistently the number of animals/cells/trials for each experiment and for each figure.

1st Revision - authors' response 18th Aug 2018

Referee #1:

Keppeler, Merino, de la Morena et al. improved an optogenetic tool, called Chronos, for stimulating spiral ganglion neurons in the ear towards the generation of an optogenetic optical cochlear implant. First, they overcame limited membrane localization by adding a trafficking signal (TS), and ER export signal (ES) leading to Chronos-ES/TS. Next, the authors have used an advanced AAV serotype (AAV-PHP.B) facilitating better optogene expression in spiral ganglion neurons. Both

improvements resulted in an advanced functional performance, carefully tested in vitro and in vivo, overcoming the poor kinetics of previously used optogenetic tools. The experiments are well performed, underlying the authors' claims that Chronos-ES/TS is superior than unmodified Chronos or ChR2. The text is clearly written, comprehensive and not overstating the results. This work clearly demonstrates the need of optimizing optogenetic tools for specific applications. In addition, the transfer and combination of previously reported modifications (ES/TS and the AAV serotype) was key to success and can be inspiring to the entire optogenetic field: Whenever expression levels are poor, one needs to tailor the optimal optogenetic tool and AAV.

First, we would like to thank the reviewer for the appreciation of our work and for the comments that helped us to further improve the MS.

Some minor points:
• Page 7/ Figure legend 2A: Does bGH refer to the pA signal? Please clarify.

Yes, it refers to the sequence coding for bovine Growth Hormone (bGH) polyA adenylation signal. We have clarified this is the legend to Figure 2A.

• Page 7 Figure 3: The data would be more convincing by adding a quantification?

Done, see Figure 3 and related section of results text.

• Page 8: Why is the data for AAV2/6 carrying Chronos-ES/TS not shown? It would be consistent to show.

Thanks for the remark. The reason is that we stopped doing those postnatal injections as soon as we realized the PHP.B enables strong transduction. Therefore, the number of animals was low (n = 2) and, in response to the reviewer's comment, we decided to not mention these experiments.

• Page 9: The sentence "Expression of Chronos seemed weaker than that of Chronos-ES/TS, but much stronger than that reported above for AAV2/6 carrying Chronos." Is not very scientific. Would it be possible to underline this finding with statistics?

We agree and have dropped the sentence. The main point is the difference in membrane abundance between Chronos-ES/TS and Chronos, which is statistically significant (Figure 4).

• Page 10/Figure legend 4F: The light grey boxes were first quite confusing that they represent the "contralateral, non-injected cochleae". It would be great to clarify this in the figure legend or figure.

Yes, done, we now used "contralateral, non-injected cochleae"

• Page 11: Have the authors really tested 120 mice for AAV2/6-Chronos?

Yes, indeed and much to our frustration.

Referee #2:

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"...suffer from a disabling hearing impairment..."

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6) Remove "But"
"But although this had been successfully employed.."

Removed sentence from introduction and provided more information in the discussion section.

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However, a number of major issues needs to be addressed before publication.

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Done, see Figure 1 and related results section

2. In Fig.2 authors present a quantification of the cellular distribution of the opsin in HEK-293T cells transfected with Chronos and Chronos-ES/TS based on the analysis of the fluorescent signal carried with confocal and STED images. The localization of the fluorescence appears more peripheral for the Chronos-ES/TS by looking at the representative images (FIG.2B) and by the analysis of the intensity profiles along lines crossing the cell membrane (Fig.2C). It is not clear and should be reported the number of line-profiles per cell and their distribution along the membranes in order to evaluate the robustness of the analysis.

We now provide further detail on the line profile analysis in the legend to figure 2 and in methods.

3. Fig.2D highlights the superior membrane expression of Chronos-ES/TS by measuring the fluorescence ratio membrane/cytosol. In this case, it should be specified over which distances and how far from the cell border have been measured the "membrane" and "cytosol" fluorescence. Moreover, also in this case, it needs to be discussed the robustness of the analysis in terms of number and distribution of line-profiles per cell. Furthermore, although the ratio membrane/cytosol fluorescence is higher for ES/TS-Chronos, it presents also a significant wider spread of data compared to Chronos. The reason for that is not clear and need to be discussed.

We now provide more detail on the analysis (see response to comment 3). The wider spread is intuitively understood as dividing by small numbers: when the intracellular fluorescence is low, the ratio gets large and small differences in intracellular fluorescence can make a big difference in ratio. We have now included the following statement:
"The larger variance of the ratio of membrane and intracellular fluorescence for Chronos-ES/TS (Figure 2D) is likely explained by dividing by the relatively low, yet varying intracellular abundance of the opsin. In summary, the data suggests improved trafficking to the plasma membrane of Chronos-ES/TS."

4. Although the analysis of fluorescence presented in Fig.2 showed superior localization of the fluorescence on the membrane for ES/TS-Chronos compared to Chronos, it appears also that the maximum fluorescence intensity at the membrane is slightly higher for Chronos than for ES/TS-Chronos (Fig.2C inset), which makes difficult to estimate the real superiority of ES/TS-Chronos in

terms of activation efficiency. In order to fully quantify the differences of activation efficiency, patch-clamp recordings of light-evoked photocurrents in Chronos and ES/TS-Chronos cells should be conducted and correlated to the fluorescence analysis.

We realize that this comparison of absolute immunofluorescence can be misleading. These experiments where not performed in parallel and did not use the same laser power or detector settings. Therefore, we would like to refrain drawing conclusions: in fact, while the estimates were higher for Chronos-ES/TS in hippocampal neurons and spiral ganglion neurons, they were higher for Chronos in HEK-293T cells. We finally removed all these comparisons from the respective figures, in order to not confuse the reader.
We think that the analysis of relative membrane abundance which is now presented for all 3 types of cells provides sufficient support of the notion of improved trafficking to the plasma membrane of Chronos-ES/TS. The other important indication for superiority of Chronos-ES/TS comes from the optically evoked auditory brainstem response data in Figure 5 which showed a significantly lower threshold for radiant flux in mice injected with Chronos-ES/TS AAV.

5. Fig.3 shows representative images of hippocampal neurons in culture expressing Chronos and Chronos-ES/TS transduced either by AAV2/6 or AAV-PHP.B. Although from those representative neurons it is observable an enhanced fluorescence localization on the membrane for Chronos-ES/TS, a statistical analysis of those results is completely missed, which makes difficult to properly quantify the improvements obtained. Again, also in this case, patch-clamp measurements of photocurrents or spike thresholds during illumination would give fundamental elements for the quantification of the entity of the improvements obtained with Chronos-ES/TS. Moreover, that would give insights on the functionality and the health of the cells.

In response to the reviewers comment we have performed further immunocytochemical analysis and provide the requested quantification. In addition, we included two more representative figures of hippocampal neurons infected either by Chronos-ES/TS or Chronos-GFP. As the focus of the study is on spiral ganglion neurons we have refrained from patch-clamp recordings from hippocampal neurons.

6. Fig.4 presents a comparison between expression in SGNs via transuterine injections of AAV2/6 and via early postnatal injections of AAV-PHP.B vector. The representative confocal images (Fig.4B and Fig.4C) of immunolabeled cochlear cryosections show an improvement in the number of positive cells in the case of AAV-PHP.B for Chronos-expressing neurons. However, a statistical analysis for Chronos AA2/6 to indicate the fraction of positive cells and their expression level would be convenient for a more exhaustive evaluation of the improvements and to make consistent some semi-quantitative claims in the text, i.e. "In most of the cases the expression of Chronos was absent" (pag7 line6); "Exceptionally, we saw high expression levels (pag7 line7)"; "confocal images suggested poor plasma membrane expression e.g. compared to Catch"(pag7 line 10).

In response to the reviewers comment we have removed most of the semiquantitative claims for the immunohistochemical analysis and put greater emphasis on comparing AAV-PHP.B transduced SGNs expressing Chronos-ES/TS or original Chronos. The oABR analysis (4 out of 120 mice showing oABR with transuterine injection of AAV2/6-Chronos-GFP) speaks for itself and the immunohistochemistry analysis is much more meaningful in the case of substantial expression (postnatal injection of AAV-PHP.B).

7. In Fig.4 Calretinin and Parvalbumin were used as generic markers of SGNs for Chronos AAV2/6 and Chronos-ES/TS AAV-PHP.B, respectively. As different markers can have a different labeling, it should be discussed how that choice can affect the estimation of the total number of cells and thus of the fraction of positive cells.

Done.

"We note that the counterstain for parvalbumin-a used for AAV-PHP.B injected ears, is a more general marker of SGNs than calretinin, used for AAV2/6 injected ears, which is only present in a subset of SGNs. Therefore, if anything, we would have been prone to overestimate the transduction rate for AAV2/6 injected ears, which, however, was very low. Interestingly, for both cases of AAV-PHP.B injection.."

8. The analysis of Fig. 4E holds the same comments previously described for the corresponding graphs shown in Fig.2C and 2D. Moreover, comparing the results obtained on HEK cells and on SGNs, it appears that the ratio between membrane/cytosol fluorescence is inferior in SGNs (Fig.4E right vs Fig.3D) and the ratio of the maximum fluorescence at the membrane between Chronos and ES/TS-Chronos presented an inverted behavior for SGNs and HEK-cells (Fig.4E inset vs Fig.3C). It would be useful to comment these aspects.

Done, we deem the analysis of Figure 4 particular telling, as the cytosolic parvalbumin stain allowed us to define the cell border independent of the opsin expression with better precision than achieved without (Figure 2). This way, panel E clearly shows the difference in Chronos localization: more membranous for Chronos-ES/TS and more intracellular for original Chronos. We have now stressed this point in the MS and also commented on the comparison to HEK cells.

"We note that the cytosolic parvalbumin immunofluorescence allowed a better estimation of the cell border which was independent of the opsin expression, both advantageous when compared the HEK-293T cell analysis (Figure 2). Despite some differences in absolute numbers between both analyses, which are not unexpected given the different cell types, means of transfection and analysis method, both support the main observation: improved relative plasma membrane abundance of Chronos-ES/TS."

9. Fig.4F and 4G analyze the transduction rate in the injected and non-injected cochleae. However, it is not clear and should be explained how the virus can spread from one cochleae to the other. Moreover, since the expression in the non-injected cochlea is not controlled, it should be explained how such an expression can be considered as a truthful terms of comparison to conclude that the injection of the virus does not cause loss of SGNs in the injected cochlea.

Done, included a sentence on the likely route for viral spread and removed the statement on the loss of neurons:
"This spread likely occurred via the cochlear aqueduct and/or the endolymphatic ducts and the cerebrospinal fluid space (Lalwani *et al*, 1996). The density of SGNs in the injected as well as in non-injected ears were comparable (Figure 5G). The injected mice behaved normal as concluded from routine animal observation."

10. Fig.6 presents an analysis of optical auditory brainstem responses (oABR) induced in Chronos- and ES/TS-Chronos-expressing mice by illuminating with "1ms light pulses delivered at 10Hz". It is not clear and should be specified the total number of pulses or the total duration of the stimulus. Also, it should be better explained the protocol of measurements, in particular the number of trials/experiments conducted per each animal to produce graphs in Fig.6B,C,E,F,H,I.

Done, 1000 trials throughout oABR. For Fig.5B,C,E,F,H,I animal numbers are reported as n within the figure.

11. Fig.6C shows oABR latencies. It is not fully clear and should be better specified how latencies have been calculated. Moreover, authors state that "oABR latency got shorter for Chronos-ES/TS and was relatively constant for Chronos". However, it seems that also Chronos latency decreases (although with a less steep slope than Chronos-ES/TS) by increasing the illumination intensity.

Latencies were calculated as time difference from stimulus onset to ABR first peak (P1) as shown in Fig.5A1,A2 and introduced in the results section. To address this point, we performed a sign test between the latency at threshold radiant flux and at maximum radiant flux (now stated in the ms). There was a non-significant trend towards lower latency for Chronos ($p=0.11$) whereas in Chronos-ES/TS the latency decreased significantly ($p=0.009$).

12. Fig.6E presents P1-N1 amplitude percentage for different illumination durations. Authors claim that oABRs could be elicited by light pulses as short as 100us with ES/TS-Chronos and 200us with Chronos. However, the plot seems to indicate the opposite, probably colors of the lines are inverted? Also it is not clear why, by incrementing the illumination durations, latencies increase for ES/TS-

Chronos and decrease for Chronos, respectively.

The wave detection algorithm was improved since initial submission and is described in the method section now. The first ABR wave is detected semi-automatically in a time window defined by the user in which the P1 is detected, if its amplitude is bigger or equal to the background signal mean + 2xSTD. This way, a P1 was first detected for light pulses as short as 20 µs and 400 µs for Chronos-ES/TS and Chronos, respectively.

13. Authors claim that "sizeable P1-N1 amplitudes can be detected up to 500Hz for Chronos and 1000Hz for Chronos-ES/". However, it is difficult to appreciate it from figure 6H because it seems that the green line corresponding to Chronos does not arrive up to 500Hz. In particular it is not clear if Chronos has been tested up to 1000Hz, as green symbols should appear even if they are equal to zero. Furthermore, error bars for Chronos are quite big and should be considered the opportunity to enlarge the statistics (n=4 for Chronos) to permit a more rigorous comparative analysis.

Statement was adjusted as suggested by reviewer. All animals were tested for up to 1000Hz. If no data point is shown in Fig.5F, we did not detect a P1-N1 wave but traces were recorded. Fig.5F was updated as suggested. In response to the reviewers request, we increased the number of experiments for both groups: by 4 and 5 for Chronos and Chronos-ES/TS, respectively, resulting in a total n of 8 and 13 for Chronos and Chronos-ES/TS, respectively. Error bars for Chronos are larger due to the generally small amplitudes being more affected by noise.

14. Fig.7 shows extracellular recordings from single putative SGN during optogenetic stimulation. Except for some circumstances (whose number should be reported in the text), mean spike probability appears to be nearly 0 for repetition rate above 500Hz (Fig.6E). Spike synchronicity with light pulses appears confined on average to around 300-400Hz (Fig.6D) and for all stimuli between 20-1000Hz the discharge rate was on average inferior to 100spikes/s. On the basis of these results it appears necessary to better discuss to which extent it can be assumed that high temporal fidelity of light-evoked spiking have been enabled and, eventually, to reformulate certain sentences (i.e., "recordings from single SGNs demonstrated high temporal fidelity of light-evoked spiking" (Abstract); "some putative SGNs were able to maintain moderate and to some extent synchronized spike rates even at pulse rates of 1000Hz"(Results, p.15); "using juxtacellular recordings we could demonstrate firing of single SGNs in response to trains of light pulses at hundreds of Hz with sub-ms temporal presision"(Discussion, p.20)).

In response to the reviewers comment we have performed further recordings. We are very excited that the additional data strongly support our claim of very high temporal fidelity: there are 16 neurons that show well synchronized responses (synchronization index or vector strength greater than 0.5) for rates of stimulation of 200 Hz, 10 for 300 Hz and 3 synchronized well to 500 Hz. While this brings a number of recordings close to what can be observed in mice with acoustic stimulation, we have nonetheless toned down the statements criticized by the reviewer. Moreover, the number of units showing a computable spike probability at rates ≥ 500 Hz are now shown in the text: "Nevertheless, some putative SGNs were able to maintain moderate (and to some extent synchronized) spike rates even at pulse rates of 1000 Hz (fibers showing computable spike probability —see Methods— at 500 Hz: 7/21, 33.3%; at 600 Hz: 3/18, 16.7%; at 700 Hz: 3/18, 16.7%; at 800 Hz: 1/19, 5.26%; at 900 Hz: 2/21, 9.52%; and at 1000 Hz 2/19, 10.53%)."

Nonetheless, we have followed the reviewers request and toned down the statement from "high temporal fidelity" to "good temporal fidelity".

15. oABRs could be detected up to 1000Hz (albeit with a very low P1-N1 amplitude), while mean spike probability of photostimulated SGNs drops below 10% for repetition rate above 300Hz (Fig.6E). It should be discussed the reasons for this difference.

Done. "The lower spike precision and limited spike probability at stimulus rates beyond 100 Hz observed are likely compensated by the population response, as several SGNs jointly encode information from each place of the tonotopic map (Liberman, 1978)"

In our experience, slower channels than Chronos-ES/TS (or less well expressed) do not support oABR at comparable frequencies (e.g. ChR2, Hernandez et al., J Clin Invest 2014; CatCh, Wrobel et

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al., Sci Translat Med 2018; Chronos-GFP at 1000 Hz, Fig. 5H this paper). Our data shows that a fraction of SGNs is able, to some extent, to follow stimulation at rates as high as 1000 Hz. We note that discharge rates at stimulation rates greater than 300 Hz and low, yet significant, vector strength may suffice to elicit synchronized neural population responses that are measurable using subdermal electrodes. This is also in accordance with our recent publication (Mager, Lopez et al., Nat Commun 2018; Wrobel, Dieter et al. Sci Translat Med), in which we report sizeable oABRs up for stimulation rates at which the average spike probability and vector strength already are very low.

Minors:

16. Please consider to have zooms for Fig.1B in order to be able to visualize the shapes of currents.

Done

17. In Fig. 4E the Y-axis title and units are missed.

Inset removed due to confusion. Absolute values not comparable.

18. In Fig.4F it should be considered the opportunity to change the title of the axis as "YFP/GFP expression" appears as a ratio, e.g. "YFP or GFP expression"

Done, changed to “FP expression (%)”

19. Fig.5 presents oABRs recorded in SGNs following transuterine injections of AAV2/6. Due to the limited number of animals exhibiting oABRs in this case (3 out of 120), it should be considered if it is necessary to have it as main figure (and in this case it should be better emphasized the relevancy of this result) or as supplementary.

Done, now Figure EV5

20. In Fig.5 it is necessary to put a temporal scale bar.

Done

21. In Fig.6 it would be convenient to show the tick marks in the logarithmic X-scale ranges in each graph to help visualization.

Done

22. It is necessary to insert in the methods an explanation for the calculation of the vector strength shown in Fig. 7C.

Done

23. It is necessary to report more consistently the number of animals/cells/trials for each experiment and for each figure.

Done

2nd Editorial Decision 24th Sep 2018

Thank you for sending us the revised version. Your study has now been re-reviewed by referee #3 and the comments are provided below. As you can see from the comments, the referee appreciates the introduced changes and support publication here.

There are just a few minor issues to sort out before we can send you the formal acceptance letter.

Please fix figure reference as pointed out by referee

We are missing 3-5 keywords

The running title is missing

Will you please check the following figure callouts and if it is OK as is or if it needs to be fixed:

Fig 3 - the panels are not called-out.
Fig 4F & G - the callouts are missing.
Fig 5F & G are called-out before Fig 5A.
Fig 5I - the callout is missing.

The figure legends + figure legends to the EV figures need to be moved to the end of the Article file.

Could you please take a look at the inserts in Figure 4C and D to make sure the inserts are to the right cells? I have looked also for Figure 2B but there I think it is OK, but please double check

The EV figures need to be changes to Figure EV1 and EV2.

Our publisher Wiley has done their pre-publication check on the manuscript has made some suggestions - see figure legends. Please incorporate their suggestions. The word file should be visible in EJP it is called Wiley pre-acceptance check. Let me know if you can't see it

That should be all. Once we get the revised version back in then I will send you the formal acceptance letter.

REFEREE REPORTS:

Referee #3:

The authors addressed my concerns and modified the manuscript accordingly. I do not have further comments and I recommend the article for publication.

Minor:

- Check figure number at pag 10: "Fig4F" instead of "Fig5F".

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Tobias Moser
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99649

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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 (eg 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 vs. unpaired), simple χ2 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 = x but not P values < x;
  - 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.

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In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample sizes used in electrophysiological cell culture experiments are the typically reported. In cell culture experiments, at least three independent replicates were assayed to ensure reproducibility.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Sample size estimations are based on experience from previous publications (Hernandez et al. 2014) and were calculated using Gpower 3.1.9.2. Details are included in the animal approval license G14.1726 and G17.2394. In the electrophysiological characterization no samples were excluded.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Animals were excluded in case of: -Otitis media -Non-repoducable ABR peaks for different radiant fluxs in a single animal -No visual reflux after virus injection (insufficient virus delivery)
3. 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.	Yes. The samples were analyzed multiple times in parallel experiments by different investigators to be sure that the analysis was unbiased. The cells analyzed were chosen randomly.
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used, but all animals were treated similar.
4.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.	No blinding was done.
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was done.
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes, normality and equal variances were tested and in case either condition was violated, non-parametric tests were used. Fig.1: NA Fig.2D,3C,4E,4F,4G: No normal distribution (KS test), Mann-Whithney U test Fig.5C: Sign test Fig.6: Rayleigh test
Is there an estimate of variation within each group of data?	Yes. Represented as standard deviation or standard error of the mean.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

6. 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., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	The following antibodies were used: chicken anti-GFP (catalog number: ab13970, Abcam, 1:500 to 1:1000 for hippocampal neurons), guinea pig anti-parvalbumin (catalog number: 195004, Synaptic Systems, 1:300). Secondary AlexaFluor-labeled antibodies (goat anti-chicken 488 IgG (H+L), catalog no.: A-11039, Thermo-Fisher Scientific, 1:200 to 1:1000 for hippocampal neurons; goat-anti guinea pig 568 IgG (H+L), catalog no. A1107, Thermo-Fisher Scientific, 1:200. Protocols were previously used in (Hernandez et al., 2014; Mager et al. 2018; Wrobel et al. 2018)
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	AAVs were generated in HEK-293T cells (ATCC) using polyethylenimine transfection (25.000 MW, Polysciences, USA) (Gray et al, 2011; Deverman et al, 2016). In brief, triple transfection of HEK-293T cells was performed using pHelper plasmid (TaKaRa/Clontech), trans-plasmid providing viral capsid PHP.B (generous gift from Ben Deverman and Viviana Gradinaru, Caltech, USA) and cis-plasmid providing Chronos or Chronos-ES/TS (Figure 1A). The cell line was tested on 24.03.2017 for presence of mycoplasma using nested PCR method. HEK-293 cells used in the electrophysiological characterization of the light-senstitive channel were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, ACC 305). Mycoplasma contamination was not accessed during these particular experiments.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All mice were maintained at the University Medical Center Göttingen (UMG-ZTE) under specific pathogen-free conditions in accordance with the recommendations of the Federation of European Laboratory Animal Science Associations (FELASA). Mice were housed in ventilated racks with integration of Individually Ventilated Caging (IVC) units in the building ventilation systems.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were approved by the animal approval number G14.1726 and G17.2394 and performed in accordance with the guidelines.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. 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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
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F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	All positive data were displayed. Negative data were reported. Data are stored on a local laboratory server and can be obtained by request.
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21. 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 format (SBML, CellML) 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 their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA