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How to make a synaptic ribbon: RIBEYE deletion abolishes ribbons in retinal synapses and disrupts neurotransmitter release

Stephan Maxeiner, Fujun Luo, Alison Tan, Frank Schmitz and Thomas C. Südhof

Corresponding author: Thomas Südhof, Stanford University School of Medicine

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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.)

Editor: Karin Dumstrei

1st Editorial Decision
12 September 2015

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

As you can see, both referees find the analysis interesting and support publication here. They raise a number of constructive comments that I anticipate you should be able to sort out. You can use the link below to upload the revised version. Let me know if we need to discuss any of the comments 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

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

REFEREE REPORTS:

Referee #1:

The authors describe here the effects of a knock-out that has been long searched for, that of the synapse-specific RIBEYE A domain. They find that the elimination of this protein results in a lack of synaptic ribbons in the mouse retina. This further induces a loss of calcium-triggered release, albeit spontaneous synaptic release is maintained.
The experiments are well-conducted, and the manuscript is well written. I only have a few suggestions to improve the clarity and/or the strength of some of the claims:
- On page 7, the authors suggest that "the RIBEYE KO abolished presynaptic RIBEYE expression without causing a major redistribution of postsynaptic mGluR6 receptor clusters (Fig 1G)." From this figure it actually seems that these receptor clusters are larger and possibly brighter in the KO synapses. The authors should check this issue.
- Figure 2 does not seem to add to the main message of the manuscript, and therefore it could be removed, to be published separately.
- On page 10, the authors claim that "the synaptic layers of the retina appeared to be generally unaltered". This claim should be supported by a quantification of the retina organization.
- The results presented in Figure 5 are somewhat puzzling. A direct interpretation of Figure 5C is that the absence of the ribbon reduces the density of the vesicles near the membrane to ~60% of the amount in the cytosol. Therefore, the elimination of the ribbon results in a selective exclusion of vesicles from the active zone area. Is this really the case? If so, how can it be interpreted? To solve this issue, it would be perhaps better to quantify the density of the vesicles near the active zones, as % of the density in the cytosol, for each individual synapse. This parameter, which would be independent of variations between synapses, could then be compared between WTs and KOs.
- One interpretation of Figures 6 and 7 could be that the ribbons also have an effect on the accumulation of calcium channels at active zones. This could be tested by an immunostaining for presynaptic calcium channels. Another issue that may be tested is whether the number of vesicles docked at active zones is similar in WTs and KOs. Clearly, the number of ribbon-attached vesicles is far lower in the ribbon-less KO synapses, which leads to lower release upon stimulation (Figure 6). But, there may be sufficient docked vesicles for spontaneous release (Figure 7). Can the authors test this by quantifying the docked vesicles in their EM images, or by perfusing the cells with hypertonic solution, to determine the size of the docked vesicle pool?

Referee #2:

This MS by Maxeiner on the effects of disrupting RIBEYE, the core component of the synaptic ribbon, provides an important and timely advance of sensory neuroscience. The synaptic ribbon is an enigmatic nanomachine in sensory cells of the eye and the ear thought to relate to the great capacity of these cells to transmit sensory information at a high rate. Previous approaches towards elucidating the function of the ribbon included disruption of its anchor to the active zone, bassoon; employing diurnal or seasonal changes of the ribbon, RNAi to ribeye in zebrafish (however not yet affecting transcripts of both genes) and photoablation. All these studies have been helpful in advancing the understanding of the synaptic ribbon. However, each of the approaches comes with its own set of disadvantages. Therefore, genetic deletion of RIBEYE is a long-awaited molecular manipulation that most directly affects the ribbon synapses of sensory cells. It also in interesting from a genetic point of view, because the manipulation needs to be selective for RIBEYE without affecting the expression of the transcriptional co-repressor CtBP2 that is expressed from the same gene as RIBEYE (which mostly worked out).

Maxeiner et al. have now managed to establish RIBEYE-deficient mice and have performed a state of the art analysis of the effects on the structure and function of retinal synapses. Impressively, there is only some reduction in CtBP2 protein levels when tested in the retina. Moreover, the authors luckily found no change in the abundance of any of the active zone proteins probed in the retina. Using paired recordings from rod bipolar cells and AII amacrine cells (RBP/AII) the authors provide good evidence that RIBEYE/the ribbon is required for both phasic and sustained synaptic transmission and further suggest that the ribbon is also involved in establishing the tight coupling of Ca2+ channels and release sites at this synapse. Finally, the authors use their KI mice to show that RIBEYE is expressed at selected nuclei of the brain, opening new avenues of research.

The genetics, morphology and physiology experiments are of high quality, the analysis sound, the writing clear and several conclusions justified and interesting. I strongly endorse publication of this paper in EMBO J provided appropriate revision.

There are a few issues that require attention during the revision of the MS.

The authors build their argument that RIBEYE/ribbons are important for Ca2+ nanodomain control on analysis of the sensitivity of spontaneous release on the slow-binding Ca2+ chelators EGTA. However, to my knowledge it is not established for the RBP/AII synapse that spontaneous and evoked release occur through the same vesicular release sites (e.g. Metha et al., 2013, 2014). Therefore, either the notion of the coupling deficit should be corroborated with evoked release or the statement be toned down.

Unfortunately, the MS has the shortcoming of nearly entirely focusing its discussion and reference on retinal ribbon synapses. Thereby, the authors miss to refer to work done in hair cells, which is highly relevant both towards the analysis of ribbon function as well as regarding the coupling of Ca2+ channels and vesicular release sites. Moreover, they also missed on the work demonstrating Ca2+ nanodomain control of exocytosis at the RBO/AII synapse. Moreover, work on the effects of photoablation and hibernation on spontaneous release at the RBP/AII synapse needs to be discussed. These references should be included in the revision of the MS.

Generally the authors might consider more referencing of original publications than of reviews.

Minor comments
Abstract:
"Here we show in mice that full deletion of RIBEYE abolishes all presynaptic ribbons in retina synapses." dependent on how many ribbon remain in the retina this statement should be revised

Introduction:
"is thought to facilitate continuous vesicle release for sustained periods (Heidelberger et al, 2005; Matthews & Fuchs, 2010)."
This is clearly restricting the current models to only the replenishment role. However, a role of the ribbon in clustering Ca2+ channels and establishing a large readily releasable pool has been proposed in the literature and this hypothesis is clearly relevant to the present study. A later discussion of models in the intro section seems uncritical and without any reference (except for the photoablation).

"In retina, the synaptic ribbon is a large, plate-like structure with a horseshoe-shaped appearance that typically appears bar-shaped in cross-sections"
This generalization does not work: it only applies to rod photoreceptor ribbon synapses.

"Ribbon synapses contain most of the components characteristic of chemical synapses," should be specified to " Retinal ribbon ...."

"These findings suggested that RIBEYE may be central to the formation of synaptic ribbons. However, morpholino knockdown of RIBEYE in zebrafish caused a significant decrease in synaptic ribbons but did not eliminate the ribbons (Wan et al, 2005, Lv et al, 2012)."
Clearly work on overexpressing RIBEYE in the zebrafish neuromast hair cells (lateral line) is relevant here.

"However, currently no manipulation is available to selectively abolish ribbons in sensory synapses,"
here or below the authors should mention/discuss insights gained from Bassoon mutants in retina and ear (including the problems)

Results:
Please note the genetic background of the mice used in the study.
Back-crossing into C57Bl/6? How many generations? Which mice were used as wild-type control:

littermate controls throughout all data sets?

Figure 1C: The upper WB image seems to be composed of several images, why?
Please comment on why two bands are visible for RIBEYE and CtBP2 and also discuss further the apparently lower CtBP2 signal in KI and KO.

Figure 1E: I am confused about the localization of the RIB-G3 protein. It seems as if it localizes only to a subset of synapses e.g. in the inner plexiform layer. Please also show the green channel in isolation for KI (e.g. splitting right panel B into two halfs or as appendix figure). Clarifying RIB-G3 localization in the retina is critical if then the authors use the KI for studies of RIBEYE expression/localization in nuclei of the brain.

Figure 1G (also see Figure 3A): There seems to be a remaining ribbon in the OPL of the KO-retina: how often was this observed in OPL and IPL, why is this (incomplete CRE-recombination?) and how did the authors ensure to record from AII's that had no ribbon? Can this be quantified?

Figure 2A-B: please add scale bars, please provide negative controls as appendix figure.

Page 9, top: "Double-labeling of sections of the laterodorsal tegmental nucleus with antibodies to EGFP (to stain for RIB-G3) and to choline acetyltransferase (to stain cholinergic neurons) revealed significant co-localization,..." please remove "significant" or provide statistical analysis

Page 9, botton: 2well as co-markers SV2 and PSD95 on vertical," please provide reference for the presynaptic localization of PSD95 in the photoreceptors.

Could the authors provide some quantification supporting in numbers the normal morphology and connectivity of retinal neurons in the KO: thickness of OPL and/or IPL. Did the authors observe abnormal neural sprouting of bipolar cell dendrites in ONL as previously observed in mice with photoreceptor ribbon synapse defects? In fact, looking at the overview image of KO retina stained for PSD95 in lower left panel of Figure 3B seems to indicate some kind of altered organization of the OPL with some terminals residing in the ONL.

Page 10 middle: "juxtaposition of the small areas of PSD95-labeled presynaptic terminals....small...." perhaps change to "small juxtapositions of ..."

Figure 4: Did the authors observe indications for synaptic degeneration? I am asking because various synaptic mutants cause some of that and I am under the impression that there may be myelin figures (eg. Figure 4B3) in the KO.

Numbers of vesicles - vesicle populations
The definitions and interpretation seems quite coarse. At least the interpretation should be weakened given the much more precise definition of structural correlates of the readily releasable pool in other studies including ribbon synapses.

"However, previously it had not been possible to test this role directly because no mutation was available that eliminated synaptic ribbons without affecting the synaptic vesicle fusion machinery."

What is meant by "affecting the synaptic vesicle fusion machinery"? Do the authors imply that photoablation affects the SNAREs or that Bassoon is part of the fusion machinery?
As stated above, I fully agree that the disruption of RIBEYE is the most direct and probably cleanest manipulation of the ribbon, however, the statements need to be more precise and if the authors implied Ca channel-release site coupling, their own data would indicate that RIBEYE disruption affects the fusion machinery.

Page 12, "Only pairs of connected rod bipolar and AII amacrine cells that exhibited stable fast EPSCs during repeated trials were included for analysis. Note that this recording strategy excludes synapses that are very weak."
Please provide an approximate fraction of recordings rejected from analysis for the reason of lacking fast transmission.

Page 13 bottom: "Interestingly, presynaptic Ca2+-currents were unchanged by all criteria in RIBEYE KO rod bipolar cells,"
Rather than showing both current and current density (somewhat redundant) in the main MS figures, the authors should consider to replace one of them by a Ca2+ current-voltage relationship.

Page 14, top: "organizing fast-sustained synaptic vesicle exocytosis at ribbon synapse" "fast-sustained" is a bit difficult as a concept
"It is of interest to note here that we found no change in release kinetics and synaptic delay (Fig 6F)." Well I would be interested in learning about the p values obtained by comparing wt and KO, there seems to be a trend in all three quantities. This is relevant as a defect in Ca2+ channel-release site coupling would be expected to show up also in the evoked release, most likely by delaying the onset and rise time.

"confirming the hypothesis that synaptic ribbons organize fast release reactions (Oesch & Diamond, 2011; Snellman et al, 2011)."
The Snellman paper actually reached the conclusion that the fast component 2011 is not affected by photoablation. However, an important function of the ribbon is likely to enabling many release sites and organizing the active zone, which would not be revealed on the time scales of the photoablation experiment. This reference should include Khimich et al., 2005 (or follow ups), which states the above conclusion in its title (for the auditory system).

"in the RIBEYE KO, the distance of the Ca2+-channels to release sites is increased."
This finding is very interesting and will likely trigger further work, such as measuring the apparent Ca2+ cooperativity in paired recordings with mechanistically different manipulations of Ca2+ influx. This paper marks a first step of this and I am fine with the scope and quantity as long as the authors carefully conclude and credit that more work is required to really test this hypothesis (see my major concern). This needs to be reflected by toning down the statements also in abstract and discussion.

Discussion
See my major concerns on balanced referencing regarding function of ribbons as learned from hair cells and Ca nano-domain coupling (both in retina and hair cells). Moreover, tone down conclusion on Ca nano-domain coupling of L-type Ca2+-channels when solely based on spontaneous release (also discussing parallel release) or do paired recordings with presynaptic application of defined concentrations of EGTA and BAPTA and measurements of the apparent Ca2+ cooperativity.

1st Revision - authors' response   31 December 2015

We thank the reviewers for their helpful and constructive comments. In the revised manuscript, we have attempted to address all of the reviewers' criticisms, and hope that the paper will now be suitable for publication in *EMBO Journal*. In the revised paper, we have made extensive changes to the text, and added new data in support of our conclusions as described in detail below. Moreover, following the recommendations of reviewer #2 we expanded the reference list and discussion of the literature significantly, resulting in an increase in the size of the overall manuscript. We hope that despite its length, the paper will still fit into EMBO J. but are prepared to cut some of the newly inserted references and discussion if absolutely necessary depending on the reviewers' and editors' opinions, and/or to transfer some of the Methods into the supplementary materials. In the following, we cite the reviewers' comments in full in black typeface, and provide our response in blue bold typeface.

Specific comments to the reviewers

Referee #1:

The authors describe here the effects of a knock-out that has been long searched for, that of the

synapse-specific RIBEYE A domain. They find that the elimination of this protein results in a lack of synaptic ribbons in the mouse retina. This further induces a loss of calcium-triggered release, albeit spontaneous synaptic release is maintained.

The experiments are well-conducted, and the manuscript is well written. I only have a few suggestions to improve the clarity and/or the strength of some of the claims:

We thank referee #1 for her/his constructive and enthusiastic comments – it is truly a pleasure to receive reviews like this!

- On page 7, the authors suggest that "the RIBEYE KO abolished presynaptic RIBEYE expression without causing a major redistribution of postsynaptic mGluR6 receptor clusters (Fig 1G)." From this figure it actually seems that these receptor clusters are larger and possibly brighter in the KO synapses. The authors should check this issue.

We agree, but think this impression might have arisen in part from the fact that the images of the different genotypes were not depicted at the same magnification. In our revision, we changed Fig 1G to fix this problem. In the revised figure, all pictures are of the same magnification in line with all later figures that show wild-type and knockout comparisons side by side. In order to evaluate whether mGluR6 receptor expression levels were changed, we performed additional quantitative immunoblotting analyses, but found no significant difference between RIBEYE KO and wild-type synapses. This result has now been added to Fig 1F.

- Figure 2 does not seem to add to the main message of the manuscript, and therefore it could be removed, to be published separately.

We agree, and have removed the data from the revised manuscript. This aspect of our study will be published in a separate study

- On page 10, the authors claim that "the synaptic layers of the retina appeared to be generally unaltered". This claim should be supported by a quantification of the retina organization.

We agree that we can only conclude that the "general" layering of the retina was unchanged by the RIBEYE KO, as documented in overviews such as Fig 2A or Fig EV3, and cannot exclude more minor quantitative changes. This has now been expressed more explicitly. Moreover, to test whether finer structural changes might be present, we used a neurofilament marker and assessed whether horizontal cell processes were still refined to the OPL. This has previously been used in models of neurodegeneration in our lab (Schmitz et al, 2006). In Fig. EV4, we are showing examples in which horizontal cell processes are sometimes found to be sprouting into the ONL, something that does not occur in wild-type retinas. We added this also in the text on page 8/9 of the manuscript.

- The results presented in Figure 5 are somewhat puzzling. A direct interpretation of Figure 5C is that the absence of the ribbon reduces the density of the vesicles near the membrane to ~60% of the amount in the cytosol. Therefore, the elimination of the ribbon results in a selective exclusion of vesicles from the active zone area. Is this really the case?

If so, how can it be interpreted?

To solve this issue, it would be perhaps better to quantify the density of the vesicles near the active zones, as % of the density in the cytosol, for each individual synapse.

We have addressed this question in an updated version of Fig 5C. Here, we display in a bar diagram the reduction found between the ratio of the number of vesicles at the active zone (rectangle) per count of vesicles in the reserve pool (black square). Since our original figure included the actual vesicle count at the active zone and of the reserve pool, we add this information as additional Fig EV5.

This parameter, which would be independent of variations between synapses, could then be

compared between WTs and KOs.

Interestingly, the reduction is in the very same range (down to 59%) as has been shown in our previous version.

-One interpretation of Figures 6 and 7 could be that the ribbons also have an effect on the accumulation of calcium channels at active zones. This could be tested by an immunostaining for presynaptic calcium channels.

We agree, and have addressed this question by quantitative immunoblotting and immunofluorescence analysis of Cav1.4 Ca^{2+}-channels. The results of the immunoblotting experiment were introduced in an updated version of Fig 1F, and demonstrate that there is no significant difference between wild-type and RIBEYE KO mice. For the IF experiments, we are presenting a new figure (Fig 3 in the revised paper since the old Fig 3 has become Fig 2 after deletion of the original Fig 2). The new IF data suggest that in RIBEYE-deficient synapses, Cav1.4 Ca^{2+}-channels are mis-localized and appear not to be aligned to the ribbon but somehow dispersed into clusters.

Another issue that may be tested is whether the number of vesicles docked at active zones is similar in WTs and KOs. Clearly, the number of ribbon-attached vesicles is far lower in the ribbon-less KO synapses, which leads to lower release upon stimulation (Figure 6). But, there may be sufficient docked vesicles for spontaneous release (Figure 7). Can the authors test this by quantifying the docked vesicles in their EM images, or by perfusing the cells with hypertonic solution, to determine the size of the docked vesicle pool?

We have counted and quantified the docked vesicles in wild-type and RIBEYE KO photoreceptor terminals. The new information is shown as a bar diagram in Fig 5D. Whereas an average of 4.9 docked vesicles per synapse were found in wild-type retinas, KO retinas showed a reduction to 1.75 docked vesicles per synapse.

Referee #2:

This MS by Maxeiner on the effects of disrupting RIBEYE, the core component of the synaptic ribbon, provides an important and timely advance of sensory neuroscience. The synaptic ribbon is an enigmatic nanomachine in sensory cells of the eye and the ear thought to relate to the great capacity of these cells to transmit sensory information at a high rate. Previous approaches towards elucidating the function of the ribbon included disruption of its anchor to the active zone, bassoon; employing diurnal or seasonal changes of the ribbon, RNAi to ribeye in zebrafish (however not yet affecting transcripts of both genes) and photoablation. All these studies have been helpful in advancing the understanding of the synaptic ribbon. However, each of the approaches comes with its own set of disadvantages. Therefore, genetic deletion of RIBEYE is a long-awaited molecular manipulation that most directly affects the ribbon synapses of sensory cells. It also in interesting from a genetic point ofview, because the manipulation needs to be selective for RIBEYE without affecting the expression of the transcriptional co-repressor CtBP2 that is expressed from the same gene as RIBEYE (which mostly worked out).

Maxeiner et al. have now managed to establish RIBEYE-deficient mice and have performed a state of the art analysis of the effects on the structure and function of retinal synapses. Impressively, there is only some reduction in CtBP2 protein levels when tested in the retina. Moreover, the authors luckily found no change in the abundance of any of the active zone proteins probed in the retina. Using paired recordings from rod bipolar cells and AII amacrine cells (RBP/AII) the authors provide good evidence that RIBEYE/the ribbon is required for both phasic and sustained synaptic transmission and further suggest that the ribbon is also involved in establishing the tight coupling of Ca2+ channels and release sites at this synapse. Finally, the authors use their KI mice to show that RIBEYE is expressed at selected nuclei of the brain, opening new avenues of research.

The genetics, morphology and physiology experiments are of high quality, the analysis sound, the writing clear and several conclusions justified and interesting. I strongly endorse publication of this paper in EMBO J provided appropriate revision.

We also appreciate the positive, extensive, and constructive comments of referee #2. Because there are so many comments, we have numbered them for easier discussion.

There are a few issues that require attention during the revision of the MS.

1. The authors build their argument that RIBEYE/ribbons are important for Ca2+ nanodomain control on analysis of the sensitivity of spontaneous release on the slow-binding Ca2+ chelators EGTA. However, to my knowledge it is not established for the RBP/AII synapse that spontaneous and evoked release occur through the same vesicular release sites (e.g. Metha et al., 2013, 2014). Therefore, either the notion of the coupling deficit should be corroborated with evoked release or the statement be toned down.

The reviewer's comment relates to two important questions in the general field of neurotransmitter release, namely whether spontaneous and evoked release are mechanistically similar, and whether the Ca^{2+}-dependence of spontaneous release (which is largely Ca^{2+}-dependent in nearly all synapses) reflects spontaneous Ca^{2+}-influx through Ca^{2+}-channels or stochastic activation of exocytotic Ca^{2+}-sensors at a resting Ca^{2+}-concentration. As discussed below, we believe that our proposed interpretation of the EGTA-chelation data applies independent of the answers to these questions, although we do agree that our explanation is a hypothesis and not a conclusion. This has now been made clearer in the revised paper. Note that performing evoked release experiments with EGTA is in fact very difficult given the need for paired recordings that would have to be stable enough for prolonged recordings.

Based on the current state of the field, it seems likely that spontaneous and evoked release are mechanistically different even though they are both Ca^{2+}-dependent and require the same membrane fusion machinery. Both for evoked and for spontaneous release, a change in the sensitivity of release to EGTA by a genetic manipulation could only be due to either a KO-induced change in the Ca^{2+}-sensor or a KO-induced change in the distance of the source of Ca^{2+} to the Ca^{2+}-sensor. Since synaptic ribbons are unlikely to have a Ca^{2+}-sensing function in exocytosis, the RIBEYE KO likely induces an increase in the distance of the Ca^{2+}-source to the Ca^{2+}-sensor.

As regards the source of the Ca^{2+} that sustains spontaneous release, a greater sensitivity of spontaneous release to EGTA in the RIBEYE KO implies that spontaneous release is normally not sensitive to EGTA, which in turn means that the source and sensor of Ca^{2+} must in very close proximity to the vesicles subject to spontaneous release – hence the hypothesis of nanodomain coupling. We show in wild-type rod biopolar ribbon synapses that although the slow Ca^{2+}-buffer EGTA has no effect on spontaneous release, the faster Ca^{2+}-buffer BAPTA inhibits it, suggesting that in wild-type synapses Ca^{2+} diffuses a short distance to stimulate spontaneous release. Most importantly, we show (also in wild-type synapses) that blocking L-type Ca^{2+}-channels suppresses most spontaneous release, identifying stochastic Ca^{2+}-channel opening as the cause for the high rate of spontaneous release in rod bipolar cell ribbon synapses. Furthermore, if spontaneous release was caused by a stochastic activation of release at the ambient resting Ca^{2+}-concentration, EGTA should have had an effect in the wild-type condition which it didn't. Viewed together, these results establish that at least for spontaneous release, there is nano-domain coupling of spontaneous release to Ca^{2+}-channels, and the RIBEYE KO impairs this coupling.

2. Unfortunately, the MS has the shortcoming of nearly entirely focusing its discussion and reference on retinal ribbon synapses.

We agree and have now included in the discussion important findings from inner hair cell studies (e.g. Khimich et al., 2005; Frank et al., 2010; Sheets et al., 2011, 2012; Jing et al., 2013; Wong et al., 2014). However, we have no data on hair cell ribbon synapses, hence the focus on retina synapses.

3. Thereby, the authors miss to refer to work done in hair cells, which is highly relevant both

towards the analysis of ribbon function as well as regarding the coupling of Ca2+ channels and vesicular release sites. Moreover, they also missed on the work demonstrating Ca2+ nanodomain control of exocytosis at the RBO/AII synapse.

We added a discussion of the study by Jarsky et al.(2010) on nanodomain coupling in RBC/AII ribbon synapses as well as some other work.

4. Moreover, work on the effects of photoablation and hibernation on spontaneous release at the RBP/AII synapse needs to be discussed. These references should be included in the revision of the MS.

We have added a discussion of the work by Snellmann et al., 2011 (p.18, photoablation) and Mehta et al., 2013, (p.18, hibernation) to the manuscript.

5. Generally the authors might consider more referencing of original publications than of reviews.

Philosophically, we completely agree that original publications should be cited. In practice, however, we are limited by the space limits imposed by the journal. At present, the references alone account for 11,500 characters of our manuscript, not even accounting for the actual discussion of these references in the text. We hope to have addressed the major concerns of the referee in regard to referencing work of others, but regrettably we simply can't give the field justice by discussing every aspect of the field because of space limitations.

Minor comments

6. Abstract:

"Here we show in mice that full deletion of RIBEYE abolishes all presynaptic ribbons in retina synapses." dependent on how many ribbon remain in the retina this statement should be revised

No ribbons remain. Ribbons are truly completely absent in the RIBEYE KO mice as documented by conventional transmission electron microscopy. In more than 200 randomly selected and documented electron micrographs of photoreceptor synapses and 90 randomly selected and documented micrographs of rod bipolar synapses, we never observed any remaining synaptic ribbon at the ultrastructural level. In contrast, in control mice normal numbers of synaptic ribbons were observed.

7. Introduction:

"is thought to facilitate continuous vesicle release for sustained periods (Heidelberger et al, 2005; Matthews & Fuchs, 2010)."

This is clearly restricting the current models to only the replenishment role. However, a role of the ribbon in clustering Ca2+ channels and establishing a large readily releasable pool has been proposed in the literature and this hypothesis is clearly relevant to the present study. A later discussion of models in the intro section seems uncritical and without any reference (except for the photoablation).

We have added further references and extended the introduction of current concepts of ribbon function. However, to the best of our knowledge no evidence exists that ribbons directly cluster Ca^{2+}-channels, while overwhelming evidence suggests that they do so indirectly via RIMs and RIM-BPs. Furthermore, the sentence cited by the reviewer was meant to include the RRP role he/she refers to – measurements of the RRP involve measurements of continuous release for sustained periods.

8. "In retina, the synaptic ribbon is a large, plate-like structure with a horseshoe-shaped appearance that typically appears bar-shaped in cross-sections"

This generalization does not work: it only applies to rod photoreceptor ribbon synapses.

Agreed and corrected in the revision.

9. "Ribbon synapses contain most of the components characteristic of chemical synapses," should be specified to " Retinal ribbon ...."

Agreed and corrected in the revision.

10. "These findings suggested that RIBEYE may be central to the formation of synaptic ribbons. However, morpholino knockdown of RIBEYE in zebrafish caused a significant decrease in synaptic ribbons but did not eliminate the ribbons (Wan et al, 2005, Lv et al, 2012)." Clearly work on overexpressing RIBEYE in the zebrafish neuromast hair cells (lateral line) is relevant here.

We added reference to the work by Sheets et al. (2011, 2012) on p.20, although the type of experiment performed by Sheets et al. is principally different from those performed by us.

11. "However, currently no manipulation is available to selectively abolish ribbons in sensory synapses," here or below the authors should mention/discuss insights gained from Bassoon mutants in retina and ear (including the problems)

We do refer to the bassoon mutants in the manuscript, but there is simply not sufficient space to discuss appropriately the entire issue of floating ribbons produced by several mutations. Since our phenotype is completely different – the RIBEYE KO abolishes all ribbons, doesn't simply dislodge ribbons – we feel that an extensive discussion of floating ribbons may also not be necessary.

12. Results:

Please note the genetic background of the mice used in the study.

Back-crossing into C57Bl/6? How many generations?

The mice underwent four to five back-crosses into C57BL/6 (note chimeras bred to C57BL/6, subsequent breeding to Flp- and Cre-deleter lines that have been from JAX and established in a pure C57BL/6 background and then removal of the before-mentioned transgene and initial expansion in C57BL/6). A sentence was added on p.21. Note, however, that having a 'pure' genetic background can also be considered a disadvantage as it renders endogenous mutations in a mouse strain homozygous, which is a major issue for C57BL/6 mice.

13. Which mice were used as wild-type control: littermate controls throughout all data sets?

We have used exclusively littermates as controls for all experiments. A statement on how we recruited the mice for different experimental procedures has been added to "Material and Methods/Generation of RIBEYE mutant mice" p.23.

14. Figure 1C: The upper WB image seems to be composed of several images, why?
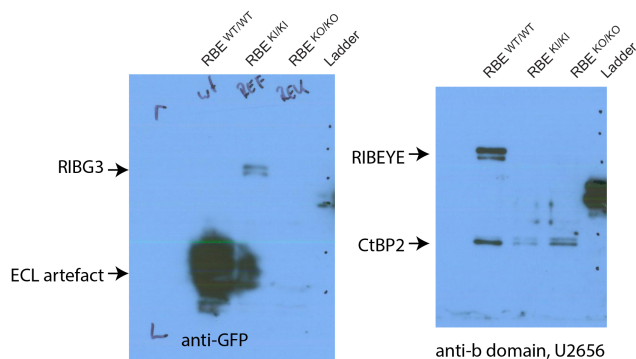
anti-b domain, U2656

The RIBEYE and the CtBP2 results are from the same blot (see original blot on left) because the antibody recognizes the b-domain and therefore both splice variants (anti b-domain, U2656). Since there is a gap between the 45 kDa band and the 110 kDa band we show this separately. The anti-GFP blot is a different antibody on a different blot using the same sample input.

15. Please comment on why two bands are visible for RIBEYE and CtBP2 and also discuss further the apparently lower CtBP2 signal in KI and KO. .

RIBEYE has previously been documented as a double band in tom Dieck et al. (2005). The two bands may arise from an unknown modification that needs to be analyzed in future studies. There is, however, no lower band in KI and KO. CtBP2 itself appears as a fine double band.

16. Figure 1E: I am confused about the localization of the RIB-G3 protein. It seems as if it localizes only to a subset of synapses e.g. in the inner plexiform layer. Please also show the green channel in isolation for KI (e.g. splitting right panel B into two halfs or as appendix figure). Clarifying RIB-G3 localization in the retina is critical if then the authors use the KI for studies of RIBEYE expression/localization in nuclei of the brain.

We modified Fig 1E and are showing both channels separately.

17. Figure 1G (also see Figure 3A): There seems to be a remaining ribbon in the OPL of the KO-retina: how often was this observed in OPL and IPL, why is this (incomplete CRE-recombination?) and how did the authors ensure to record from AII's that had no ribbon? Can this be quantified?

We believe that this image was showing cross-reactivity or autofluorescence that was observed for some but not all CtBP2 or RIBEYE antibodies (especially monoclonals). The possibility of incomplete CRE-recombination is excluded because these experiments were performed on constitutive RIBEYE KO mice in which the gene has been Cre-recombined in the germline, as described in the manuscript.

We now provide an updated version of Fig 1G in which we adjusted the magnification of both image sets and excluded the cross reactive spot. We also added quantitative electron microscopic data that further supported the proposal of complete absence of retinal synaptic ribbons in RIBEYE knockout mice.

18. Figure 2A-B: please add scale bars, please provide negative controls as appendix figure.

We have removed Fig 2 following referee #1's suggestion.

19. Page 9, top: "Double-labeling of sections of the laterodorsal tegmental nucleus with antibodies to EGFP (to stain for RIB-G3) and to choline acetyltransferase (to stain cholinergic neurons) revealed significant co-localization,..." please remove "significant" or provide statistical analysis

Deleted upon removal of Fig 2.

20. Page 9, bottom: 2well as co-markers SV2 and PSD95 on vertical," please provide reference for the presynaptic localization of PSD95 in the photoreceptors.

We added the reference to the paper by Koulen et al., 1998, on p.9.

21. Could the authors provide some quantification supporting in numbers the normal morphology and connectivity of retinal neurons in the KO: thickness of OPL and/or IPL. Did the authors observe abnormal neural sprouting of bipolar cell dendrites in ONL as previously observed in mice with photoreceptor ribbon synapse defects? In fact, looking at the overview image of KO retina stained for PSD95 in lower left panel of Figure 3B seems to indicate some kind of altered organization of the OPL with some terminals residing in the ONL.

Immunolabelling with SV2 and PSD95 and also the EM data showed a close to normal layering of the retina. Immunolabelling with anti-neurofilament antibodies that label horizontal cell processes revealed some moderate sprouting of horizontal cell processes into the outer nuclear

layer (ONL). Thus, while the overall architecture appears to be largely normal, we cannot exclude minor structural changes as a consequence of reduced synaptic signaling, e.g. from photoreceptor ribbon synapses. We incorporatd a comment about our observation on p.9 and added Fig EV4.

22. Page 10 middle: "juxtaposition of the small areas of PSD95-labeled presynaptic terminals....small...." perhaps change to "small juxtapositions of ..."

We amended the cited sentence.

23. Figure 4: Did the authors observe indications for synaptic degeneration? I am asking because various synaptic mutants cause some of that and I am under the impression that there may be myelin figures (eg. Figure 4B3) in the KO.

Please see our comment above. Anti-neurofilament immunolabelling showed some discrete sprouting of horizontal cell processes into the outer retina. This sprouting is moderate in comparison with retinas with strong neurodegeneration, e.g. in CSPα knockout mice (Schmitz et al., PNAS Proc Natl Acad Sci USA 103: 2926-2931)

24. Numbers of vesicles - vesicle populations

The definitions and interpretation seems quite coarse. At least the interpretation should be weakened given the much more precise definition of structural correlates of the readily releasable pool in other studies including ribbon synapses.

Please, refer to our answer to referee #1. We updated Fig 5 in regard to docked vesicles.

25. "However, previously it had not been possible to test this role directly because no mutation was available that eliminated synaptic ribbons without affecting the synaptic vesicle fusion machinery."

What is meant by "affecting the synaptic vesicle fusion machinery"? Do the authors imply that photoablation affects the SNAREs or that Bassoon is part of the fusion machinery?

The sentence explicitly refers to mutations, not photoablation. Again, space constraints do not allow a detailed discussion of photoablation approaches but the data on its use appear to suggest that it cannot eliminate synaptic ribbons without causing photodamage to surrounding structures.

26. As stated above, I fully agree that the disruption of RIBEYE is the most direct and probably cleanest manipulation of the ribbon, however, the statements need to be more precise and if the authors implied Ca channel-release site coupling, their own data would indicate that RIBEYE disruption affects the fusion machinery.

We don't quite agree – our data show that the RIBEYE KO affects neurotransmitter release at ribbon synapses, not necessarily the fusion machinery. Indeed, the fact that spontaneous release is normal in RIBEYE KO synapses under control conditions strongly suggests that the fusion machinery is normal.

27. Page 12, "Only pairs of connected rod bipolar and AII amacrine cells that exhibited stable fast EPSCs during repeated trials were included for analysis. Note that this recording strategy excludes synapses that are very weak."

Please provide an approximate fraction of recordings rejected from analysis for the reason of lacking fast transmission.

Only 1 out of 10 RIBEYE KO recordings was excluded because of an unstable EPSC. In general, the success rate for achieving a good pair-recording was approximately 25-40% for both groups (n=5 mice for both genotypes), with no obvious difference between wild-type and RIBEYE KO retinas.

28. Page 13 bottom: "Interestingly, presynaptic Ca2+-currents were unchanged by all criteria in RIBEYE KO rod bipolar cells,"

Rather than showing both current and current density (somewhat redundant) in the main MS figures, the authors should consider to replace one of them by a Ca2+ current-voltage relationship.

We have not performed I/V measurements for Ca^{2+}-currents as there is no reason to assume changes in properties of Ca^{2+}-currents in a situation where the amplitude and density of Ca^{2+}-currents are unchanged. Showing both is useful because they only partly overlap – the fact that they are both unchanged means that the overall Ca^{2+}-current kinetics is not grossly altered.

29. Page 14, top: "organizing fast-sustained synaptic vesicle exocytosis at ribbon synapse" "fast-sustained" is a bit difficult as a concept

"It is of interest to note here that we found no change in release kinetics and synaptic delay (Fig 6F)." Well I would be interested in learning about the p values obtained by comparing wt and KO, there seems to be a trend in all three quantities. This is relevant as a defect in Ca2+ channel-release site coupling would be expected to show up also in the evoked release, most likely by delaying the onset and rise time.

The p-values were p>0.05. The reviewer is correct that in a classical chemical synapse an increased distance between Ca^{2+}-channels and release sites should have manifested as a change in the timing and reliability of release, but our experiments do not allow conclusions about this prediction. Testing this prediction using action-potential induced EPSCs (which may or may not be physiologically relevant for bipolar cells since these neurons are generally considered to be non-spiking) is beyond the scope of the current manuscript.

30. "confirming the hypothesis that synaptic ribbons organize fast release reactions (Oesch & Diamond, 2011; Snellman et al, 2011)."

The Snellman paper actually reached the conclusion that the fast component 2011 is not affected by photoablation. However, an important function of the ribbon is likely to enabling many release sites and organizing the active zone which would not be revealed on the time scales of the photoablation experiment. This reference should include Khimich et al., 2005 (or follow ups), which states the above conclusion in its title (for the auditory system).

The reviewer naturally is correct, but Snellman et al. still conclude that ribbons organize release reactions. We have now also cited Khimich et al. (2005).

31. "in the RIBEYE KO, the distance of the Ca2+-channels to release sites is increased."

This finding is very interesting and will likely trigger further work, such as measuring the apparent Ca2+ cooperativity in paired recordings with mechanistically different manipulations of Ca2+ influx. This paper marks a first step of this and I am fine with the scope and quantity as long as the authors carefully conclude and credit that more work is required to really test this hypothesis (see my major concern). This needs to be reflected by toning down the statements also in abstract and discussion.

We naturally agree that more work is needed, and hope our paper will be the first step towards such work. However, we feel that our conclusion cited by the reviewer is fully justified by the evidence we present as discussed above and now more extensively in the revised paper, and as also based on outstanding work in the in literature using similar approaches.

32. Discussion

See my major concerns on balanced referencing regarding function of ribbons as learned from hair cells and Ca nano-domain coupling (both in retina and hair cells). Moreover, tone down conclusion on Ca nano-domain coupling of L-type Ca2+-channels when solely based on spontaneous release (also discussing parallel release) or do paired recordings with presynaptic application of defined concentrations of EGTA and BAPTA and measurements of the apparent Ca2+ cooperativity.

In the revised paper, we have either provided a more extensive explanation for our conclusions, or toned down these conclusions as recommended by the reviewer.

Acceptance	01 February 2016

Thank you for submitting your revised manuscript to The EMBO Journal. Your revision has now been re-reviewed by the original referees.

As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the manuscript for publication here.

Congratulations on a beautiful study!

REFEREE REPORTS:

Referee #1:

The authors have replied convincingly to my comments, and I am happy to suggest publication of the manuscript.

Referee #2:

The authors have addressed most of my concerns and while I do not fully agree with all responses I overall support publication of this version of this exciting MS in EMBO Journal.

EMBO PRESS

Corresponding Author Name: Thomas C. Südhof & Frank Schmitz	
Journal Submitted to: EMBO Journal	
Manuscript Number: EMBOJ-2015-92701	

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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.

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.

B- Statistics and general methods

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	To study survival and comparision of Mendelian ratio we used the numbers of mice that have been genotyped and met the criterion to be offspring of heterozygous breedings. For Immunoblotting and qRT-PCR it is established in the field that an N of 4 is sufficient to assess any statistical significance. Our selection of age matched mice and littermates as well as processing all samples at the same time (for qRT-PCR, IB and IF), we aimed to limiting variations that may arise from technical handling. For electrophysiology, our sample sizes were determined based on historical requirements-at least 6 cells from 3 animals for each group.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	See 1.a. for qRT-PCR, survival studies, IB and IF. For electrophysiology, no statistical methods were used to predetermine sample sizes, but our sample sizes are similar to those generally reported in the field.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The data were excluded for analysis purely based on the cell health and quality of patch-clamping of recording.
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.	N/A
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used for electrophysiology recording. Rod bipolar cells and AII amacrine cells were selected for recordings based on their morphology, stratum location in the retina and electrophysiologica parameters.
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.	Yes. The mice for immunoblotting, qRT-PCR and imaging have been genotyped and grouped without specifing prior to the experiment which was which. Furthermore, samples of each group that have been used for immunoblotting and qRT-PCR were run alternatingly.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Mice were ear tagged and genotyped and grouped by a person that was not doing the actual experiment to assure that experiments were done blindly.
5. For every figure, are statistical tests justified as appropriate?	In figure 1 we are presenting results on animal survival compared to Mendelian distribution. It is well accepted in the field that this is done using the CHI-square test. In all figures that are depicting the comparision of transcript levels (qRT-PCR) or protein levels (immunoblotting), we applied a Student's T-Test. Yes. The statistics used are found in each figure legend. Data distribution was assumed to be normal but this was not statistically tested.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We applied the tests that are well accepted in the field to assess any differences between the two groups of genotypes.
Is there an estimate of variation within each group of data?	Yes. Data are reported as mean +/- SEM.

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).	All antibodies that have been used in this study are listed respectively in the material and methods section of our manuscript, see "antibodies" p.27. We specified the usage (immunoblotting or IF), the clone number and catalogue numbers.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* 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 the information requested has been listed in the respective section of "material and methods/generation of ribeye mutant mice", pp.21/22.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	We have included the statements in "material and methods/generation of ribeye mutant mice", pp.21/22
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.	We comfirm compliance.

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
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and 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.	NA
17. 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.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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).	NA
20. 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 agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. As far as 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** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. 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

23. 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.	The use of TTX has been approved refer to "All animal procedures conformed to National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Stanford University Administrative Panel on Laboratory Animal Care (IACUC committee)." p.22.