Reviewer 2:
I disliked the organization of this paper, the numerous overstated claims and the lack of appropriate quantification reported in the text/figures.

We disagree that our data are overstated. We did provide quantification for every motif in the original text. The reviewer failed to read it.

We have also developed new open-source Tulip query software so that anyone can perform regular expression (regex) queries to extract the true numbers of each motif. The dataset on which this extraction is performed is now a time-stamped supplemental file RC1-20160515-nw-ALL.tlpx . This is explained in the methods and text. With this tool we now added all the motif data in RC1 instead of just our traced examples.

We reported all the motifs we had traced and those numbers were in the original text.

For example, we reported n=144 for C1 motifs on page 12. That is now n=152 on page 14 since we have traced 8 more instances. That is a large sample compared to other connectomics papers in retina and brain. However, we neglected to make clear that these are the motifs we validated by human visualization. We actually have many more C motifs discovered by database queries in RC1. Table 3 shows the total number of C motifs found in RC1 to be 1225. The total number traced by intent is 262.

We had more-than-sufficient data originally, reported it and have now augmented it with database search numbers.

1a. Quantification – The paper purports to quantify the number and types of crossover inhibition among BCs. However, the results section is filled with statements that are not supported by even the most basic reporting requirements such as number of each motif type observed. I suppose readers are supposed to look this up for themselves in the CRXO.xls file?

Reply: The numbers for every motif class were in the body of the original manuscript.

1b. This file is cited in the text but I had to request the file.

Reply: Apologies. Upload failure.

1c. The paper is sorely in need of one or more summary tables that clearly list the total numbers of cells traced, whether those cells are completely or incompletely traced, and the number of motif types observed. Without this information clearly communicated to the reader, many of the results appear anecdotal at best.

Reply: We have complied. In addition to the numbers originally cited in the text, we have added Tables 2 & 3 that summarize data instances, and changed the supplemental table to add up the traced instances and added new class instances to the supplementary data. We had presumed, incorrectly, presenting the data in the text (as is

traditional) was adequate, especially especially for R motif data. Now each class number is replicated four times: in the text (as in the original ms), in two new tables, and the supplementary table.

2a. Based on the XLS file, the numbers of motifs are C1 = 147, C2 =9, C3 = 6, C4 = 70, C5 = 4, CR = ?, R1 = 35, R2 =35, R3 = ?, R4 = ?, R5 = ?, R2/4 = ?. There are numerous question marks and empty entries in the file, it's not clear what to make of these.

Reply. See above.

Also note that the *.xls file was intended (as stated in the original text) to list BC instances associated with the major C motifs, to list all classes of BCs involved with crossover C-R crossover, and provide index numbers for navigation. It did that. A second point is that connectomes are growing datasets continually in revision as more annotations are made. The file thus contained many annotator comments. We have removed most of those (they are still in the connectome in the property metadata for every cell), and added the sums that were already in the text.

2b. The authors claim to be able to distinguish BC subtypes, so why are some listed as question marks?

Reply: We hadn't fully annotated every bipolar cell that every motif contacts so we didn't know all assignments. And the ? refers to processes leaving the volume, not cell class. This changes nothing in the structure of the paper since all CBb classes are involved. Tables 2 and 3 re-emphasize that the definition of each motif does not depend on cone BC class but includes all classes. We stated that in the original abstract, body and discussion.

2c. More problematic is the missing information for multiple motifs (CR, R3, R4, R5, R2/4).
Reply: All included. The numbers were in the original ms text. See above.

2d. Do the authors really believe the few observations of motifs C2, C3, and C5 are sufficient to claim these are significant?

Reply: Absolutely, for three reasons (1) Connectome structure and the nature of cell patterning; (2) Low density cells are important; (3) No cases of rare cells that have ever been proven chimerical.

1. Connectome structure:
As we have previously shown and discussed in many papers (PMID 19855814, 21118201, 21311605, 22498714, 23042441, 24016532, 25237297), connectomes sample a canonical space of any tissue. Davi Bock also briefly mentions this in his Nature paper on their cortical connectome (PMID 21390124). Class-wise sampling must reflect the densities of the underlying cells, their coverage factors (e.g. see Wassle

PMID 480198), Hausdorff dimensions and their joint distribution samplings (Marc PMID 24016532. Cells with large fields, low soma densities, coverage factors of 1-2 (minimally overlapping dendrites) such as ganglion cells and polyaxonal amacrine cells will have low representation rates in a 0.25 mm diameter connectome. So such cells will necessarily only have 1-10 copies in the entire connectome (e.g. C3, R3, CR). Connectome RC1 contains only one OFF alpha ganglion cell, which is correct for its size and eccentricity. It contains only one one interstitial amacrine cell, which is correct for its size and eccentricity. These are all known low coverage factor cells. So the fact that we have found crossover motifs in this superclass of low density cells is pretty impressive to us. However, cells with massive coverage factors such as wide field ACs that make up motifs C1, C4, R1, R4 would be expected to have 100s to 1000s of copies (based on coverage factors published since the 1990s. Those expectations match our data. The coverages of glycinergic narrow field cells are poorly known (except for AII amacrine cells … see Marc PMID 25237297) and appear to be very low in RC1, and their volume joint distributions with bipolar cells are much lower than wide-field cells, explaining why motifs C2, C5, R2, R5 have lower instances. We briefly explained this originally and have touched up the ms to re-emphasize it.

2. Low density cells are important.
The senior author showed in 1977 that the primate fovea contains 3% blue cones and 10% in the periphery (Science, 197&, PMID 403607). This in no way diminishes the power of blue percepts. Blue-ON GCs are even rarer and an RC1-like connectome of parafoveal primate retina would contain no more than one such cell. Its connectivity is presumed to be unique however and useful even so. The Substantia Nigra contains ≈ 750,000 cells, less than 1-10% of the density of cells it controls in striatal and thalamic targets. The motor impact of Parkinson's Disease is well known, despite the miniscule innervation involved. A connectome of caudate would contain maybe one or two SN dopaminergic terminals.  One would still report those data. Alpha ganglion cells make up less than 1% of the ganglion cells in the retina, but no one suggests they are ineffective. Indeed, the connectivity of the single alpha GC in RC1 is consistent with its known physiology and coupling. The same arguments can be garnered for aminergic systems and opioid systems in retina and brain: low density, high impact. Low numbers are <u>expected</u> for certain cell classes.

3. No chimeras.
We are not aware of a discovery of a rare cell and motif that turned out to be ephemeral. We would be quite interested in an example from the reviewer. Please note that much of the work on invertebrate connectivity, such as the crustacean stomatogastric network involves one copy of each cell per animal.

Even though individual cell classes may be rare, simple sampling theory predicts that that they are found at a rate matching true density. In 1977 Kolb and West (PMID 853310) described a unique interplexiform cell in the cat retina that was later argued to have GABAergic markers. The connectivity of that cell is known from only one instance from the Kolb lab and <u>maybe</u> one from the Sterling lab. That's it. No one suggests that these rarities should be neglected or unpublished. Our own work on very low density

glycinergic interplexiform cells in teleosts (Nature, 1984, PMID 6504139) shows a unique pattern of connectivity whose visualization is enabled by molecular markers. So the low numbers, in our view, reflects density. We would be happy to be shown wrong

2c. What is the threshold of number of observations to consider a particular wiring pattern a motif?

Reply: See above and Tables 2 and 3. Technically one. If you find a motif, report it and describe it. The alternative is not acceptable. We've discussed low density cells and joint distributions in many papers.

2d. Even more puzzling, the Discussion states that 'C4 and C5 motifs are several fold more abundant that C1 or C2'. It isn't clear how the authors came to this conclusion, again highlighting the need for a more transparent reporting of basic numbers.

Reply: The synaptic densities underlying that claim were listed in the original text. We have amplified that to make it clearer. As we stated originally, Every rod BC gets about 10 C motif inputs. Every AI AC gets about 100. We say it again and again include the data. We have provided exact counts for multiple AI cells instead of just the summary data. The result is the same.

3a. Motif C1: This is one of the few places in the paper that the authors at least perform some basic quantification in the text. The authors claim all cone BCs classes drive C1 GABA-ACs, please list the numbers, sorted by BC class, in table form instead of requiring the reader to look this up in a XLS file.

Reply: Since C1 motifs engage all BC classes and since some contact different classes at pre and postsynaptic sites, a simple sorting by class isn't possible. The reviewer has unwittingly suggested a classic and difficult combinatorial query problem. The resulting table is unmanageable and unnavigable. We summarize all the encounter instances for all CBb classes in the text. Database query tool are provided for any users.

3b. For long range C1 motifs (up to 0.25mm), what, if anything, are the GABA-ACs synapsing on in between rod BCs?

Reply: They contact cone BCs as described in the original text, as shown explicitly in the *.xls file and as illustrated in the figures.

4. Motif C2: The authors estimate that each rod BC receives 1 or 2 C2 motifs. Why is this an estimate? What are the actual numbers based on what was observed?

Reply: It is an estimate because we haven't reconstructed all paths to all BCs. That would be impossible. We based it on the the five rod BCs we did reconstruct and the C2 motifs we found. Our original statement is absolutely correct. We augmented it now to read: "A regex query of RC1 shows that all C2 motif paths found so far make either 1

(13 cases) or 2 synapses (1 case). We thus estimate that every rod BC receives only 1 or 2 C2 motif synapses."

5a. OFF BC motifs: The authors make the italicized statement that cones can drive inhibition through every AC synapse on a rod BC terminal. This seems way over-stated.

Reply: Not at all. It is an accurate statement of what our motifs show.

5b. The authors acknowledge in the Discussion that it is not clear that the proximal inhibitory inputs to large AI ACs could actually electrotonically effect their distal varicosities providing inhibition to AII ACs.

Reply: We were being gentle. It was a citation, not an acknowledgement of fact. We did explicitly say that our data are inconsistent with that model and why we think that claim by Grimes et al are wrong. We have increased our explicit opposition.

6. Motif C3: How many GABA-AC C3 motifs were observed?

Reply: Six. The original text said six.

7a. The statement that 'our connectomics resolution (2 nm) ensures we do not miss any synapses (Marc 2014b)' is fairly grandiose. The authors seemingly are ignoring the fact that they are using 70-90 nm sections which is no longer state-of-the-art in the connectomics field.

Reply. Not grandiose at all. We don't agree that the reviewer gets to define the connectomics field. We are absolutely correct and the reviewer is absolutely incorrect. TEM images are projection images. The structures inside them are visible in the Abbe electron map. This was known in 1950. That's why goniometry, tomography and TEM phase imaging work. At 80 KeV the internal step resolution is 270 pm. The average synaptic size in our volume is >100 nm, a factor of 370 fold. When projected on to a 2nm grid, the Z-axis is irrelevant. The goniometric projection equation predicts we should see every synapse and gap junction.

We are ignoring no facts. The section thickness is optimal for this task. We discussed this in detail in 2011 (Anderson et al., PMID 21118201). In that peer-reviewed paper We explicitly analyze serial sections through near tangential synapses and state that the reconstruction prom projection images cannot be solved by making slices thinner. We also explain why SEM ablation techniques require thinner sections since the secondary scattering equation predicts (and everyone's results show) no section penetration. Combined with bad SEM lateral resolution, this is a problem, not a state-of-the-art advance. We are, and have been, correct on this point from the beginning. Please note that TEM has long been used in retinal serial section imaging (Kolb, Sterling, Meinertzhagen, Fisher).

7b. At a minimum, numerous gap junctions are likely missed when they are not captured in cross-section.

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Reply: Incorrect. We routinely capture oblique gap junctions (no one else has shown can) and have also showed goniometric tilt corrections in PMID 21311605. Peer reviewed.

Furthermore, the reference supporting this dubious claim is a book chapter that was presumably not peer-reviewed.

Reply: see above.

8. Following on the above point, the authors should include a description of their minimal criteria for annotating synapses. Were synapses observed in multiple sections? Several of the example images of ribbons appear questionable at best: Fig 7B, 10C, 12C,D, 16D. This exemplifies the problem with a ~40x lower Z resolution than lateral resolution that is unacknowledged in the paper. Since the authors claim perfect synapse annotation, they should report how they cross-validated results between human annotators. How many false positive/negative detections were made? The network analysis presented later in the paper suggests they annotated one false positive connection. Is this only one error out of 1.2 million annotations? Similarly they should address how many neurites could not be successfully traced due to the anisotropic sampling of the data.

That's a different topic we have published on previously and we suspect the reviewer knows this. This is completely irrelevant to this manuscript.

9a Motif C4: How many AI ACs were analyzed to arrive at the 60-100 synapse number?

Reply: This was in the manuscript.

9b. What does the statement 'Most of the instances we have been successful in tracing…' mean? What limits successful traceability?

Reply: Time and annotators. We are a small lab.

9c. What supports the claim that C4 synapses onto AI ACs are 'among the very largest inhibitory synapses in the retina',

Reply: The data originally cited in the paper. Fig. 20. Supplemental table 2 (that the reviewer did not request)

9d. have the authors also extensively sampled AC synapses onto GCs?

Reply: Yes. That's a separate paper. AC synapses onto GCs are not significantly different in size than AC synapses onto BCs and other ACs. It is not relevant to this discussion. But the data in the original paper comparing synapses onto AI ACs and OFF BCs from the same motifs are correct and illustrate the point.

10a. Motif C5: Isn't the estimate that inhibitory synapses outweigh rod BC excitatory inputs by 9:1 heavily biased by the limited size of the data volume?

Reply: No. Because each rod BC also has all AI AC inputs that come from outside the volume. It is based on the number of synapses each rod BC gets, unaffected by the volume's geometry and inputs they come from.

10b. Is this a useful estimate if most of the long range AI AC dendrites that receive rod BC synapses extend beyond the dataset?

Reply: See above.

11a. General question: Are the various GABA-ACs and GACs unique to each C motif or are some ACs shared by multiple motifs? Based on the presentation, it is difficult to discern this.

Reply: We explained this in the original manuscript. We have repeated it in new Table 2.

11b. The presentation also does not address whether there is any cone BC class specificity that contributes to each C motif. Were the motifs analyzed in a BC class dependent manner?

Reply: Since the reviewer had the *.xls, we presume they could see the obvious answer to this is yes. C1 motifs are not class specific. We said so in the original manuscript.

12. Motif CR: The dendrite of GABA-AC 20299 appears to be less than 100 um long? Was the rest of this cell not traced for some reason?

Reply: 20299 originated outside the volume. It has been traced further through the volume, but doesn't appear to leave it. This doesn't change its assignment.

13. Motifs R1/R2: Are the different cone BCs involved in these circuits of the same cone BC class?

Reply: No, as described in the Tulip section (that the reviewer below deemed not useful). All these networks are mixed. None are pure. A full analysis of this and what it means is a separate paper.

14. It would help if the figure presentation style was somewhat standardized. Some figures show IPL stratification profiles of the reconstructed cells, others do not. I suggest

adding stratification profiles to all motif examples.

Reply: The images are very standardized as they are and include the views that most compactly make our points. Stratification views do not help the analysis of crossover.

15a. Motif R5: What fraction of rod BC dyads synapse onto AI/AII vs. GABA-AC/AII? The statement that 'a few rod BCs could suppress 60-100 cone BCs' seems like wild speculation.

Reply. As stated in original ms "In particular, cell 18282 spans the entire RC1 volume" and such cells are "likely spiking neurons that could spread signals as wide as their dendritic fields (> 0.5 mm in diameter)."

So it is pretty simple. If 18282 is a 0.5-1 mm diameter cell, its true area is 4-8x the size of RC1. 4x-8x the number of BCs contacted by 18282 (13, as stated in the original ms) = 52-104 . So it is not a wild speculation at all.

We've renamed this motif R3 (so C1 matches R1, C2 matches R2, C4 matches R4 and C5 matches R5).

15b. A few sentences above, the GABA-ACs participating in R5 are indicated to also receive input from 'many' ON cone BCs. It isn't clear that the sparse rod BC inputs are strong enough or densely enough spaced to initiate spikes (absent cone BC activity) in the GABA-ACs.

Reply: No one knows yet. But note as above in 15a, this is just part of the cell's field, so it likely gets 4-8 rod BCs. Target load says nothing about what the source strength needs to be. Ever. Most wide-field ACs spike, as shown many times by many physiological studies. And R3 is only one of six R motif paths.

16. Motif R2/4: How many examples of each of the four ON-OFF GAC classes were observed?

Reply: We have renamed this motif R25 (explained in the ms). We only have a single complete copy of each variant. But they all are in class R25, n=4. We made this diversity of cell classes within a motif clear for motif C1, where we explicitly stated that many different cell types contribute to this motif. We don't have multiple copies of every submotif yet. A few years of work coming.

17. None of the network topology figures are particularly helpful in demonstrating any of the conclusions. For example, the finding that most ON cone BC 2-hops occur within class, but many are between class is impossible to read off of Fig 19D. It seems like a simpler accounting of the different motifs encountered, as suggested above, would be far more useful.

Reply. We disagree. See question 3. Making a list is not so simple and it is not clear to us what point the reviewer is making. The graph is an outcome anyone can reproduce in Tulip and further analyze on their own. That is too extensive for an already long paper.

18. The final two summary figures are helpful, but this would have been a great place to indicate the frequencies of each motif observation.

Reply. The table has been added.

19. Methods: BCs are described as patches of coupled cells, do the authors mean electrically coupled? If so, please specify.

Reply: Coupling by gap junctions was made explicit early in the paper. We added it again.

20. Methods: The authors should clearly state what they mean by an 'annotation'. Does that mean contouring a cell profile in one section, identifying one synapse, etc…?

Reply: Our annotation methods have been extensively discussed in previous peer-reviewed publications e.g. PMID 21118201, 21311605, and others, all of which were cited in the Methods.