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September 3, 2020

Dear Dr. Upponi,

We are very pleased to re-submit our paper, entitled "Behavioral assessment of visual function via optomotor response and cognitive function via Y-maze in diabetic rats," for publication in the *Journal of Visual Experiments*.

We thank the editors and reviewers for their insightful comments that have strengthened the manuscript. We are pleased that the reviewers thought the OMR and Y-maze descriptions "could be useful for researchers working in different fields". We have made the requested revisions to the manuscript based on the editors' and reviewers' suggestions and have addressed the comments in a point-by-point fashion below, with changes throughout the manuscript in tracked changes.

We hope that the manuscript is now acceptable for publication.

Sincerely,

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Editorial Comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Response: Thank you. We have carefully proofread the manuscript for spelling and grammar errors.

2. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

Response: We have sorted the table alphabetically and included all equipment. Our OMR comes as one whole system, and the company does not use catalog numbers.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Thank you. We have adjusted all text to remove personal pronouns.

4. Please use the generic name for Quatricide instead of the brand name.

Response: We have changed Quatricide to "sanitizing solution" throughout.

5. Please do not abbreviate journal titles in the references.

Response: Thank you. We have spelled out all journal names.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Good job. I will be interested in seeing the video.

Major Concerns:

- I would suggest removing no-brainers from the method description such as plugging the power strips etc.

Response: Thank you. We have removed this from the methods description.

- Discussion in row 337-342. Albino subjects are known of their bad vision, due to many factors including disturbed binocularity, lack of melanin in the back of the eye and large proportion of dual opsin cones. Please emphasize this point that albinos may not be good subjects for OMR testing because their performance may be too close to limit of detection.

Response: We agree that this is an important point and have modified the discussion as such:

"Meanwhile, some strains of albino rats seem to have compromised spatial frequency[22], while other strains of albino rats do not exhibit tracking behavior at all. Many factors may contribute to the limited response of albino animals on OMR: disturbed binocularity due to differential decussation of optic nerve fibers, lack of melanin in the back of the eye, and large proportion of

dual opsin cones. Regardless, albino rats may not be appropriate subjects for OMR testing since their performance could be too close to the limit of detection."

- I would appreciate if authors could give guidelines if contrast sensitivity or spatial frequency threshold testing is better in respect to diabetes and maybe also retinal degenerations models? How about rotation speed? Temporal frequency?

Response: This is a great question, and something we wonder about as well. I am hesitant to say that particular aspects of OMR are more sensitive for a particular disease. I personally see SF change earlier in diabetic retinopathy models, but others in the lab have seen CS change earlier – and that is with the same model in the same lab. I just don't think we have definitive data on this.

- In OMRs alternative methods the authors do not mention electrophysiology at all, even if it may be the most precise way of detection visual dysfunction. ERG (however not with pattern stimulation) is also much more available than OMR. Please see and refer to e.g. Leinonen & Tanila 2018, Behav Brain Res.

Response: Thank you for the suggestion. We have modified the discussion and added this reference.

"Electrophysiological assays are an alternative to behavioral visual tests. Electroretinography (ERG) is more available than OMR and can determine deficits in precise cell types using different components of the ERG wave (a- waves represent photoreceptor cell function, b- waves represent bipolar cell function). Meanwhile, OMR can be used to determine a deficit visual function, without revealing the precise point of breakdown along the pathway. However, OMR is a more sensitive measure of DR than ERG, with OMR deficits typically observed between 2-4 weeks post hyperglycemia and ERG deficits typically observed 4-8 weeks post hyperglycemia in rodents."

- I would like if authors could very clearly state their opinions/recommendations of the:
1. Time of the day when OMR and Y-maze would be best performed
2. Duration of a single trial
3. How many trials per day is ok, and at what intervals?
4. Cumulative daily time.

Response: Good questions! We have added the following to the discussion:

OMR*: "Running the rats before noon appears to be best for their focus (Rachael Allen's lab – personal observations). If rats become too distracted, it can help to gently tap on the outside of the OMR.*

The speed with which the testing is performed can also affect results. Measures may become less accurate after 30 minutes or so if the rodents lose interest in the stimulus. Therefore, more accurate results may be obtained when measurements are taken in approximately ≤20 minutes. Duration of a single trial (for either SF or CS) is 5-10 minutes for an expert and 30 minutes for a beginner. If a rodent is showing little movement, spending most of its time grooming, or otherwise not looking in the direction of the bars, it may be fatigued. The rodent may be run again on a different day. Additionally, SF and CS testing can be performed on different days, particularly for newer testers who may be slower. The frequency with which the test is performed can also affect results – performing it weekly or every other week helps the animals stay acclimated to the test, but performing it every day or every other day can cause hyperacuity[24]. We do not run more than one trial per day, though we often run both SF and CS in the same day or even the same sitting. Cumulative daily time for running a cohort of rats (n=10) is 2 hours for an expert."

Y-maze: "*It is also recommended to run the Y-Maze at the same time each day to account for changes in activity levels throughout the day due to circadian rhythms. We typically run the rats before noon (Rachael Allen's Lab – personal observations). Duration of a single trial is 8 minutes (10 minutes, with clean up). We never run more than one trial per day. If an additional trial is needed, the trial is performed on another day. Cumulative daily time for running a cohort of rats (n=10) is 2-3 hours."*

Minor Concerns:

- Row 70: retina may be more vulnerable than what? Other tissues of the body?

Response: We have edited this to say, *"than other neural tissue[12]."*

- Row 91-92: This is just a stylistic matter, but are parentheses really needed here? I think text would flow smoother without.

Response: We have removed the parentheses.

- Row 94-95: I do not understand what authors are trying to say here. What about OCT and ERG? As if something is missing here.

Response: Thank you for catching this. We have adjusted the text so that it reads as such:

"Both are relatively sensitive, in that they can be used to detect deficits early in the progression of diabetes in rodents, and reliable, in that they produce results that correlate with other visual, retinal, or behavioral tests. Additionally, using OMR and Y-maze in conjunction with tests such as electroretinogram and optical coherence tomography scans can provide information on when retinal, structural, and cognitive changes develop relative to each other in disease models."

- Row 358: OMR scoring without automation IS subjective, not only CAN BE.

Response: Thank you. We have made this change.

- Row 361: A well functioning automated OMR has been developed and is commercially available (qOMR by Phenosys) and should be mentioned here.

Response: Thank you for the suggestion. We have added the following sentence but cannot state the company by name due to the journal's policy (we also do not state the companies who make our equipment by name).

"As of 2016, an automated or quantitative OMR system called qOMR has been well developed and is commercially available."

- Rows 399 and 401 - Weinshenker and Allen labs. Please give also first names. There is too many Allen last named PIs to make a distinction.

Response: Thank you. We have made this change.

- Figure 4. I am curious. Why is there such strong interaction? Why is exploratory behavior so high in GK rats initially?

Response: We are honestly wondering the same thing. The reviewers for that paper asked us to run an additional cohort that began at 4 weeks instead of 5 weeks, so the small cohort of rats run at 4 weeks (GK: n=7, Wistar: n=10) is the only group we have ever run at that timepoint. I'm reluctant to speculate on why it happens until I can be sure we see it again.

- Figure 5. ERG retinal = "electroretinography retinal". I suggest to put it: ERG/retinal function

Response: Thank you. We have adjusted this figure.

Reviewer #2:

Manuscript Summary:

The aim of the present report was to describe protocols for OMR and Y-maze tests in rats. In addition, the authors show the performance differences for diabetic and control rats in these tests. I think that the report could be useful for researchers working in different fields. However, there are some points that should be addressed before publication,

Major Concerns:

Introduction:

…. a tissue that may be more vulnerable to oxidative stress and metabolic strain from diabetes. More vulnerable than what? Please, rephrase

Response: Thank you. We have edited this to say, *"than other neural tissue[12]."*

Regarding this paragraph: Clinically, the relationship between retina and brain has been studied in the context of Alzheimer's Disease and other diseases but is not commonly explored with diabetes15; reference 15 refers exclusively to Alzheimer's Disease, to address other diseases (as mentioned in the text), other references should be added.

Response: We have included additional references for this statement.

Results

Regarding data shown in Figure 3 and 4, no information about the statistical analysis is provided. In addition, is highly confusing the number of animals (10-23?, 7-29?) used in Figure 4. It is not clear the meaning of the P value, particularly which groups are being compared. These are important issues. The authors should mention how many animals/group should be used to find significantly statistic differences among groups, and they should suggest the proper way to analyze the data from a statistical standpoint.

Response: Thank you for these important points. The issue with the number of animals is due to the reviewers for that paper asking us to run an additional cohort that began at 4 weeks instead of 5 weeks. We have added the following to the figure legend for Figure 4 as an explanation:

"Only one cohort of rats was run from 4 weeks to 8 weeks (GK: n=7; Wistar: n=10). All other cohorts were run from 5 weeks to 8 weeks (GK: n=22; Wistar: n=23) for a total n of 29 (GK) and 33 (Wistar) at weeks 5 through 8."

We have added the following information on statistical analysis to the results section:

OMR: *"Data were analyzed using a one-way ANOVA followed by Holms-Sidak post-hoc comparisons as the young and aged results came from different cohorts."*

"Data were analyzed using a two-way repeated measures ANOVA followed by Holms-Sidak post-hoc comparisons. 6-10 animals, depending on severity of injury, are typically needed to find a significant difference with OMR."

Y-maze: *"Data were analyzed using a two-way repeated measures ANOVA followed by Holms-Sidak post-hoc comparisons. A minimum of 10 animals, depending on severity of injury, are typically needed to find a significant difference with Y-maze."*

We have also added information to the figure legends about which groups are being compared with regard to p values.

For OMR, have the authors noticed differences between gratings moving in a clockwise and counterclockwise direction? Please, comment

Response: Thank you. We have added the following sentence to the discussion:

"In control rats, there should be no difference in results between clockwise or counterclockwise directions, but some rodents could have a bias so it would be best to alternate the directions if the OMR system does not alternate automatically."

For models causing differential eye damage (e.g., glaucoma), have the authors noted any influence of the damaged eye on the undamaged eye?

Response: Thank you. We have added the following sentence to the discussion:

"OMR measures each eye independently, resulting in separate visual scores for each eye. In the Morrison and microbead models of glaucoma and in an optic nerve crush model, our lab has not observed any impact of the damaged eye on the undamaged eye. In a blast model, with the blast directed at one eye, the contralateral eye did show damage, but this could also be due to a partial blast effect."

Please, make any comment on the inter-individual variability in normal and diabetic animals. Based on the data shown n Figure 3, it seems that this parameter (i.e., SEM) could be significantly different among groups. I believe that some "biological information" is hidden behind this issue.

The protective effect of exercise in diabetic rats regarding SF seems unclear (Figure 3A). In contrast, the exercise seems to be effective regarding the contrast sensitivity. Thus, the lack of correlation between the SF and the CS should be discussed. In addition, the authors should explain to potential readers the biological meaning of SF and CS.

Response: This is an important point about the SF results with exercise. When additional cohorts were run with active animals and active animals + a TrkB inhibitor, the SF and CS results did correlate, as published in Allen et al., 2018. We have decided to remove/replace Figure 3A – partly for this reason and partly because we wanted to include a new SF figure that shows strain and age differences with raw SF numbers for reference.

For the point about the biological meaning of SF and CS, we have added the following sentence to the introduction:

"Spatial frequency refers to the thickness or fineness of the bars, and contrast sensitivity refers to how much contrast there is between the bars and the background (Figure 1E)."

In Figure 4, it seems that control rats changed their performance in the Y-maze along the study (i.e, the spontaneous alternation seems higher at 8 than at 4 weeks), whereas the exploratory behavior seems to go in the opposite direction. As for diabetic GK rats in the same test, differences in spontaneous alternation and exploratory behavior along the study are dissimilar. Is there any rational explaining why exploratory behavior is much more sensitive to diabetic progression than spontaneous alternation? Please, check the intra-group statistic variability for normal and diabetic rats along the study.

Response:

Cognitive and exploratory behavior for control rats (added to results section): *"Control rats seem to show a decrease in exploratory behavior over time. This trend is also observed in long term studies (8+ months of age). The decrease in movement could be due to lack of novelty with repeated maze exposure or a general decreased movement with age. Control rats appear to show an increase in spatial cognition over time. This trend is* not *observed in long term studies in which the animals are run monthly instead of weekly (in fact, a decline with aging is often observed), and thus, this increase in spatial cognition may be due to a learning effect of running the maze once a week."*

Exploratory behavior for diabetic rats: We are honestly wondering the same thing about why the 4 week exploratory behavior is so high for the GK rats. The reviewers for that paper asked us to run an additional cohort that began at 4 weeks instead of 5 weeks, so the small cohort of rats run at 4 weeks (GK: n=7, Wistar: n=10) is the only group we have ever run at that timepoint. I'm reluctant to speculate on why it happens until I can be sure we see it again. If you ignore the 4 week timepoint, the exploratory and cognitive behavior trends are more similar.

How much can diabetes-induced locomotor impairment affect spatial cognition in the Y-maze test? Do the authors consider it necessary to independently evaluate locomotor activity in diabetic rats, before the Y-maze test?

Response: This is an interesting question. We have addressed it as such in the discussion:

"While both exploratory behavior and spatial cognition decrease in diabetic rodents, the two do not appear to be tightly correlated, and thus, we do not independently evaluate locomotor activity prior to Y-maze testing."

What do the authors mean by "The scores for individual animals were of sufficient quality"?

Response: Good question. By "sufficient quality", we mean that an animal entered at least five arms on Y-maze or that an animal did not have trouble paying attention on OMR, resulting in a falsely low score. We explain this elsewhere in the paper, and worry this wording is confusing here, so we have removed it.

The age-influence (if any) on the performance in the Y-maze test should be discussed.

Response: Thank you. We have added the following to the discussion:

"Age-related decreases in spatial alternation were observed in rats at 9-12 months of age and in exploratory behavior at 12 months of age."

How might the incidence of diabetic cataract affect the results in the OMR test?

Response: Thank you. We have added the following to the discussion:

"Severe diabetic cataracts can affect OMR. However, diabetic cataracts in rodents appear and/or worsen under anesthesia, and thus, tests like ERG and optical coherence tomography that require anesthesia are affected much more often that OMR, which is performed in awake animals."

Minor Concerns:

Please, define STZ, OCT, ERG, SF, CS at their first use

Response: Thank you. We have defined these terms at first use.

Instead of: "is used to ablate pancreatic beta cells", it should be: is used to damage pancreatic beta cells

Response: We have made this adjustment.

Which signs should be considered to visualize rodent fatigue?

Response: Thank you. We have added the following to the discussion:

"If a rodent is showing little movement, spending most of its time grooming, or otherwise not looking in the direction of the bars, it may be fatigued. The rodent may be run again on a different day. Additionally, SF and CS testing can be performed on different days, particularly for newer testers who may be slower."