February 27, 2015

Dear Editor,

Please find enclosed the edited manuscript in Word format (file name: 15973-Revised).

Title: The importance of being Nernst: an essential role for synaptogenesis and network emergence as higher-order metrics of neurogenesis in stem cell-derived neuron models

Author: Aaron B Bradford and Patrick M McNutt

Name of Journal: *World Journal of Stem Cells*

ESPS Manuscript NO: 15973

We appreciate the considered feedback from the reviewer and editor, and feel that the resulting changes have strengthened the manuscript. The following changes were made at the request of the editor:

1. Format and font has been updated.

2. Running title, contact information, telephone #, author contributions, conflict of interest statement, and core tip have been added to the text of the manuscript

3. Disclaimer has been added indicating source of training funding for Bradford AB

5. Minor grammar and spelling corrections

6. References format has been fixed, including addition of PMID and DOI numbers to all references

7. Figures have been updated to allow more deconstruction

The following revisions have been made according to the suggestions of reviewers (highlighted in yellow in the revised manuscript):

Reviewer 1 comments to the authors

(1) "I recommend including a table (or expanding Table 1 of the manuscript) with the studies the authors think should be emphasized to the general readership, including the studies' results and methodological strengths"

Table 1 includes all studies that we could find which applied synaptic activity measurements (of variable rigor) to neurons derived from all species other than mouse (there are *many* studies showing synaptic activity in mouse e-neurons). We have excluded a wide array of studies that did not incorporate such analyses. Table 1 has been further updated to include strengths and weaknesses of each technical approach; the timeframe at which activity was detected; and the nature of the observed activity.

(2) "The abbreviation mGRASP should be explained. Also in this section (Approaches for morphological and structural characterization of neurogenesis) the differences and the time course of physical and

functional synapse formation should be addressed."

The abbreviation mGRASP was explained and a functional definition of the method was added. We expanded on the process of synaptogenesis, the meaning of synaptic apposition versus function and three different methods to characterize apposition in the section titled "Approaches for morphological and structural characterization of neurogenesis". However, the comparable rates of apposition versus function are context-dependent (e.g., types of neurons, age of neurons, culture conditions, whether dendritic spines are involved, etc.) and such studies are generally poorly time-resolved, so we are not sure there is a simple answer to this request. We could not find a good characterization in SCNs.

(3) "I would recommend expanding on the time course of the appearance of minis in human pluripotent stem cell derived neuronal populations. After how many days of differentiation should minis be expected in the most important differentiation protocols?"

The timepoint at which activity was reported as well as the type of activity was added to Table 1. However, which protocols are the most "important" is still very much in question; even for those models that develop minis, it is not clear that most (or possibly any) human i-neurons ever develop robust synaptic and network activity to date. We would not be surprised if the problem is the pluripotency status of the human iPSC lines that are currently being used (see the brief mention we make of 'ground-state' or 'naïve' iPSCs in the section titled "Induced pluripotent stem cells").

(4) "The use of calcium imaging and genetically engineered calcium indicators (GECIs) could/should have more emphasis in the article. Although shortly mentioned, these studies may directly demonstrate synaptic coupling and significantly extend electrophysiology data in this regard."

The reviewer is correct that Ca^{2+} imaging and related techniques have extensive utility on their own and as a complement to electrophysiology. Although calcium imaging does not (currently) have the spatial or temporal resolution of electrophysiology, with the appropriate pharmacological manipulations, similar functional conclusions can be drawn regarding synaptically driven activity. We have added considerable material discussing the strengths and limitations of Ca^{2+} imaging and the transformative potential of optical electrophysiology in the section titled "Network behaviors as higher-order signatures of neuron function".

(5) "Is there a suggestion for researchers to carry out the investigation of plasticity-related immediate early genes in developing neuronal cultures? What are the possibilities for using paired electrophysiological recordings in culture? Do microfluidic chambers offer an opportunity to this end?"

We have lengthened the section titled "Activity-dependent responses as an indirect measure of synaptic function" to focus on the value and limitations of using plasticity-related gene expression as a complement to functional readouts in neuronal cultures. Since many of these genes are also involved in other developmental and cellular behaviors, it is important that their expression be specifically associated with conditions that elicit or impair synaptic activity without causing other stress. We also have included a paragraph in the section titled "Synaptic activity is a functional signature of successful synaptogenesis" discussing the value and limitations of paired recordings in dissociated neuron cultures. Potential applications of microfluidic chambers are now highlighted as a future approach in the section titled "histogenic models: a step beyond networks".

(6) "Is there any method to accelerate functional synaptogenesis in vitro? Is overexpression of specific transcription factors a feasible option for this method?"

This would be an excellent topic to cover as it appears this is a goal of many researchers. We have

included a paragraph in the “Induced pluripotent stem cells” section discussing the use of transcription factors and small molecules to accelerate differentiation and/or improve the efficiency of differentiation in iPSCs.

(7) “Are the authors aware of any study successfully using planar multielectro [sic]”

This statement was cut off in our version of the reviewer’s comments. However, we have included a multi-paragraph discussion of the potential and limitations of using MEAs and calcium imaging to study network activity in the section titled “Network behaviors as higher-order signatures of neuron function”. Furthermore, we include a brief mention of several instances in which MEAs were used to monitor longitudinal responses to neuromodulatory conditions in the section titled “In vitro neurotoxicology”.

Again, we would like to thank the reviewer for valuable suggestions, and the editor for considering this manuscript for publication in the *World Journal of Stem Cells*.

Sincerely yours,

Aaron B Bradford, PhD
Patrick M McNutt, PhD