Response to reviewers' comments (Manuscript # JoVE60295)

1. Response to editorial comments

General:

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

Response: The revised manuscript has been properly proofread.

2. *Please ensure that the manuscript is formatted according to JoVE guidelines–letter (8.5" x 11") page size, 1-inch margins, 12 pt Calibri font throughout, all text aligned to the left margin, single spacing within paragraphs, and spaces between all paragraphs and protocol steps/substeps.*

Response: We have formatted the manuscript as per the above guidelines.

3. *JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.*
For example: Accutase, TrypLE, Milli-Q, Sharpie.

Response: We have removed reference to Milli-Q, Sharpie. We have replaced Accutase with 'acutase cell dissociation solution" (page 3, step 1.4). We have retained the use of "TrypLE" as it is the dissociation reagent recommended for optimal use of GABAergic interneurons and control endothelial cells.

Protocol:

1. *There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headers and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.*

Response: Filmable protocol has been highlighted in yellow. Some of the steps post-cell seeding are common in all three assays. We have highlighted these steps when mentioned first (in the long-distance migration assay) to adhere to the 2.75 page limit.

2. *For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to*

perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

Response: We have adhered to these guidelines in the revised manuscript.

Specific Protocol steps:
1. *1.2.1/1.3.1: What should the cell density/confluence be here?*

Response: We have mentioned the percentage cell confluency required at these two steps. (page 4, step 3.1; page 5, step 4.1).

2. *3.5/4.5: Please explain what proper seeding looks like.*

Response : By "proper seeding' we meant cells that adhered properly and there was no leakage from the insert compartment during overnight seeding. We have included this in the revised manuscript (page 6, step 6.5; page 7, step 7.6; page 8, step 8.7).

Figures:
1. *Please remove 'Figure 1' etc. from the Figures themselves*.

Response: We have removed the texts "Figure 1", "Figure 2", "Figure 3" etc. from the actual figures.

2. *Define the scale in the appropriate Figure Legend.*

Response: We have added scale bar for all microscopic images and defined the scale in the figure legend.

3. *Figure 2E, 2F, 3C: The legend mentions mean ± S.D., but there are no error bars.*

Response: We have added error bars in the revised graphs.

References:
1. *Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.*
2. *Please do not abbreviate journal titles.*
3. *Please do not include references that have not been accepted yet (the current reference 10).*

Response: We have formatted the references and excluded reference #10.

Table of Materials:
1. *Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.*

Response: We have updated the Table of Materials and provided information on all materials and equipment used.

Response to comments from Reviewer #1:

In this manuscript, the authors proposed protocols for three in vitro assays - the long-distance migration assay, the co-culture assay and the chemo-attraction assay - using co-cultures of paraventricular endothelial cells with or without human induced GABAergic interneurons.

These assays may allow for live cell time-lapse imaging which offers an advantage over conventional cell migration assays such as Transwell systems that only display the end result of the migration process. However, there are some concerns need to be addressed:

Response: We thank the reviewer for appreciating the advantage of our assays.

Major points:

1. *It will be nice to include a chart to compare and contrast assays described in this manuscript with other existing relevant assays.*

Response: We have included a chart (Table 1) that compares the features of our assays with those of other relevant assays that are commonly used.

2. *The authors need to provide more information on 1) the origin, stage, and subtypes (eg. SST or PV) of iGABA neurons, 2) contents of culturing media, 3) any adverse effect of mixed medium on the growth and morphology of endothelial cells and iGABA neurons, 4) the origin of control endothelial cells, and 5) how they optimized the seeding density of iGABA neurons and endothelial cells.*

Response:

1) We have purchased the human GABAergic neurons from a commercial vendor (mentioned in NOTE, step 3, page 4). These are derived by differentiating a human fibroblast-derived iPSC line. These neurons have been subtyped for regional and neural markers by the company and are available from them upon request. We have not characterized these cells further for SST, PV subtype specificity.
2) We have provided complete information on the content of culturing medium of human periventricular endothelial cells (page 3, step 1.1).
3) For GABAergic interneurons and control human endothelial cells, culture media are purchased from the vendor (mentioned respectively in NOTE, step 3, page 4;

NOTE, step 4, pages 4 and 5). The information is also listed in Table of Materials.

4) Adverse effects of co-culture medium on neurons and endothelial cells have been tested by cell viability assay and morphology study using immunocytochemistry. This has been mentioned in the discussion of our revised manuscript (page 11).
5) Control endothelial cells are obtained from the differentiation of a human fibroblast-derived iPSC line. Further information on the source of fibroblasts or differentiation method is available from the vendor.
6) Seeding density of GABAergic neurons were as per manufacturer's recommendation (information provided in NOTE, step 6.1, page 6). The seeding density of control endothelial cells and human periventricular endothelial cells were followed to maintain 1:1 ratio with seeded neurons (mentioned in NOTE in step 6.2, page 6).

3. *The authors may use simple plots instead of 3-D plots in Figure 2, 3, and 4.*

Response: We thank the reviewer for this suggestion. We have changed our graphs and used simple bar graph plots in the revised version.

4. *The authors need to revise the sentence on page 4 "paraventricular endothelial cells were co-seeded with the GABA-interneurons to mimic the conditions in vivo", which seems to be an overstatement.*

Response: We have rephrased this sentence (page 2).

5. *It is not clear why Figure 1C was included.*

Response: Original Figure 1C was included to show the outlined rectangular patch inside which cells are seeded. We have included a better image to represent this in the revised Figure 1. This is mentioned in the text (page 6, step 5.10) and in revised figure legend for Figure 1 (page 10).

6. *The authors may want to check apoptotic cells during the migration assay.*

Response: We have confirmed absence of apoptosis in the cells by staining with anti-active Caspase 3 antibody. Data has been included in revised Figure 2E and mentioned in the Representative Results section (page 9) and in Figure legends for Figure 2 (page 10).

7. *Size bars are missing in Figures 1,2C and D, and 3B.*

Response: We are sorry to have missed the scale bars. We have added scale bars for all microscopic images and defined the scale in the figure legends.

8. *How the authors normalized percentages of cells in Figure 3C? Also, it looks like less than 3% of cells migrating in Figure 2F. What was the criteria for cell migration?*

Response: We have changed the original plot in Figure 3C. In the new graph, we have removed the normalization and plotted the graph as relative number of neuronal cells that have migrated towards periventricular endothelial cells or control endothelial cells.

In Figure 2, we have re-represented cell number versus distance migrated as a single graph (in Figure 2F). For clarity and consistency, in this graph too we have plotted migrating neuronal cell number versus distance travelled.

Minor Concerns:
Not applicable.

Response to comments from Reviewer #2:

Manuscript Summary:
This protocol provides a good resource as to assay the interneuron migration affected by periventricular endothelial cells, and can be easily applied to monitor migration of many other cell types.

Response: We thank the reviewer for the positive remarks.

Major Concerns:
None.

Minor Concerns:
1. *It is better to clarify (and state the detailed steps) as to whether and how the differentiated periventricular endothelial cells can be regularly passaged and stored before performing the assays.*

Response: We have added an extra section in protocols detailing the steps for routine culture and freezing of human periventricular endothelial cells. This is the new step 1 (pages 3, 4).

2. *Line 116: before detach cells by adding Accutase, should the medium in the wells be removed first?*

Response: The medium needs to be removed first before adding Accutase. We missed this step, and thank the reviewer for pointing it out. We have added this step in the revised manuscript (page 3, step 1.3).

3. *Line 125, Line 134: before detach cells by adding TrypLE, should the medium in the wells be removed first?*

Response: We have added this step in the revised manuscript (page 4, step 3.3; page 5, step 4.3).

4. *For Steps 1 to 3 (page 5), it is better to put more detailed steps about how the authors prepare Matrigel, poly-L-ornithine/laminin, fibronectin coated plate, respectively.*

Response: We have provided more detailed steps on preparation of Matrigel (page 3, step 1.2) and poly-L-ornithine/Laminin (page 5, steps 5.1 and 5.3).

We purchased control human endothelial cells from a company (mentioned in Table of Materials). We followed manufacturer's recommended protocol for culturing these cells on Fibronectin substrate. Fibronectin coated plates were prepared according to manufacturer's protocol. In the revised manuscript, we have omitted the mention of fibronectin-coated plates. Instead, we have referred to follow the manufacturer's protocol for preparation of fibronectin coated plates 9 (mentioned in NOTE following step 4, page 5).

5. *The numerical order of the steps could be confusing:*
Page 4: 1. Cell Preparation
Page 5: 1. Preparation of human periventricular endothelial cells
Page 5: 3. Preparation of control human endothelial cells
Page 6: 2. Preparation of One-well Culture Inserts
The author should put the numerical orders in a better way.

Response: We have changed the numbering order. The new format consists of major steps 1, 2, 3 and sub-steps 1.1, 2.1, etc.

6. *Line 176, 178: what kind of medium is used?*

Response: For neuron dish, neuronal medium is used, and for endothelial cell dish, endothelial cell medium was used. We have revised the line and added information on the kind of medium in the revised manuscript (page 7, step 6.7).

7. *Line 213, 214: it is better to link the text to Figure 3A here as well.*

Response: We have linked this step to Figure 3A (page 8, step 8.4).

Response to comments from Reviewer #3:

Manuscript Summary:
The manuscript by Dutta and Vasudevan provides new assays to assess the interaction between specialized human iPSC-derived endothelial cells and interneurons.

Response: We thank the reviewer for commenting on the novelty of the assays.

Minor Concerns:
***1.** While the assays are described well and in details that can be reproduced, the manuscript can benefit from a better introduction of the endothelial subtypes in brain and their function both during development and in adult brain.*

Response: We have provided information about the different endothelial subtypes in the embryonic forebrain and their distinctive features (page 2).

2.*Similarly, the discussion could include potential effects of these endothelial cells on brain cells other than interneurons*,

Response: We have discussed the potential effects of these endothelial cells on neural progenitors and projection neurons (page 11).

3. Typos and grammatical mistakes should be checked.
Line 30….vasculature in nervous system
Line 43… alludes to
Line 69… patterns

Response: We have corrected these mistakes in the revised version.

4. lines 74/75…. Using the stem cell technology….should this be iPSC technology….

Response: We have replaced “stem cell technology” with “iPSC technology” (page 2).

5. please add a brief 1-2 lines description of the technology used in the lab that was previously published.

Response: In-depth protocol for generation of human periventricular endothelial cells has been developed in the lab, and is part of a manuscript that is currently undergoing peer review.