**Rebuttal letter**

**REVIEWERS COMMENTS:**

**Reviewer #1:**

Manuscript Summary:

The experimental method, in utero transduction, provided by David Lutz is of great scientific value for neurodevelopment and neurological diseases. However, several specific experimental steps and experimental results need to be further clarified or supplemented.

We thank the reviewer for the helpful suggestions.

Major Concerns:

1. In 4.1 Mapping the area of interest

About "At 85 °C, the Araldite resin becomes trimmable". It is a fact that the resin is easy to be trimmed at high temperatures. However, it is easy to squeeze the sample at high temperatures and damage or deform the ultrastructure. It is recommended to use a trimming machine (e.g. Leica EM TRIM2) or sandpaper or thin blade to trim sample at room temperature.

We are grateful for this comment and we have now included the recommendation to use a trimming machine, thin blade or sandpaper within this protocol step.

2. about "Figure 3 and Figure 4"

Figures 3 and 4 are the results of the method in the present study, and they need to be rearranged. Since the semi-thin section is 0.75μm, it would show a higher resolution. Based on low magnification, please add the same pictures under high magnification (in 60X, 63X, or 100X Objective) in optical microscope. The electron micrographs are very beautiful at high magnification, and please add the same electron micrographs at low magnification with large field of vision.

We thank the reviewer for appreciating the quality of our images. We have followed the suggestions and included semi-thin microphotographs (100x objective) as well as low magnification TEM of mossy fiber boutons in the hippocampus and cerebellum, and spinal cord. We have rearranged Fig. 3 accordingly. We have deleted the schematic representations of mossy fiber boutons. As suggested by reviewer 2, we have also provided a high magnification of the PSD in Fig. 4D. We would like to point out that Fig. 4 is thought to contain only examples of synaptic boutons from other species at TEM level for comparative purposes, as the focus of the manuscript lies on the mouse. We have therefore omitted overloading the figure with detailed representation of semithin sections.

Minor Concerns:

1. In 1.2.2 Surgery

During "Inject the AAV1-Fast-Green mixture under visual inspection of the dye penetration", need a stereoscope to accurately position the injection site?

We thank the reviewer for this suggestion and we have now indicated the use of a stereoscope during the injection.

2. In 2.1 Perfusion of mice

The references are too old and are older than 50 years old from 11-16. I read some of them and the experimental methods were outdated. Please add 3-5 Please add or update references in recent years (e.g. Liu J, Liu B, Zhang X, Yu B, Guan W, Wang K, Yang Y, Gong Y, Wu X, Yanagawa Y, Wu S, Zhao C. Calretinin-positive L5a pyramidal neurons in the development of the paralemniscal pathway in the barrel cortex. Mol Brain. 2014 Nov 18;7:84. doi: 10.1186/s13041-014-0084-8.).

We understand the concerns of the reviewer. We have cited the original works that were essential for the establishment of our method. We have also followed the suggestion to include the work by Liu et al. 2014.

3. In 2.1.2 Mugnaini's fixation solution (5 L)

About "Note: Before fixation, warm Mugnaini's fixation solution to 40°C!". Afer " Before fixation", it is best to add a step for perfusion with saline or phosphate buffer (Mol Brain. 2014 Nov 18;7:84. doi: 10.1186/s13041-014-0084-8), which is a key step in perfusion to remove blood cells and plasma, otherwise the cyclic perfusion would fail. For a beginner, it is easy to skip this step.

We thank the reviewer for this helpful comment. We have now included two essential steps: Heparin infusion and perfusion with phosphate buffer before perfusion with Mugnaini’s fixative.

4. In 2.2 Embedding into agarose, documentation, and sectioning It is best to use Low Melting Point Agarose, which can be glued at around 30 ° C and melted at about 65° C. 45° C may damage the ultrastructure of the tissue (Thermal expansion).

We thank the reviewer for the suggestion and we have now included the use of low melting agarose and of agarose embedding at 30°C.

5. In 3.2 Dehydration of tissue and interference reflection light imaging About "100 % propylene oxide (30 min) at room temperature". It is best to note that propylene oxide treatment is 2 times to completely remove ethanol. If there is ethanol residue, the sample would not polymerize well.

We agree with the reviewer and have revised the manuscript to include 2x30 min of propylene treatment.

6. In 3.3.2 Araldite/propylene oxide (1:1) solution It is best to add a step of "Araldite/propylene oxide (1:2) solution" to completely remove ethanol propylene oxide.

We have now added a step of 1:2 Araldite/propylene incubation in the protocol.

7. In 3.3.2 and 3.3.3

I think that there should be no different between "Mix Araldite basic stock and 3 % accelerator" and "Mix Araldite basic stock and 2 % accelerator". The two steps could be unified to simplify experimentation.

We thank the reviewer for the suggestion. We have now unified both steps to one.

Reviewer #2:

Manuscript Summary:

The authors clearly demonstrated the method of zoom-in from the optical microscope level to the electron microscope level with the brain tissues affected by virus vector transduction in utero. This kind of technology is useful for many researchers. The method is described in detail, it is high in quality so that anyone can reproduce it.

We thank the reviewer for the positive evaluation of our method.

Minor Concerns:

In figure 4D, some PSDs (\*) are unclear. The authors should replace the image, change the contrast, or show high-maginication photo(s) to show PSD(s) more clearly.

We have now followed the suggestion of the reviewer and show high-magnification TEM of a PSD in Fig. 4D.