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Assessment of ultrastructural neuroplasticity parameters after in utero transduction of the developing mouse brain and spinal cord

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To

Dr Alisha DSouza

Science Review Editor
(JoVE)

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"Assessment of ultrastructural neuroplasticity parameters after *in utero* transduction of the developing mouse brain and spinal cord"
by Lutz *et al.*

Dear Dr DSouza,

Thank you for sending us the editorial comments on the manuscript. We have addressed all editorial concerns and revised our manuscript accordingly. We have appended the track-changed manuscript with the answers to the editorial comments as a separate rebuttal letter file. The changes in the table of materials are indicated in red. A separate rebuttal letter that contains our reply to the reviewer comments is also appended.

Best regards,

David Lutz

TITLE:

Assessment of Ultrastructural Neuroplasticity Parameters After In Utero Transduction of the Developing Mouse Brain and Spinal Cord

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KEYWORDS:

mossy fiber synapse, spinal cord, hippocampus, cerebellum, myelin lamellae, cell adhesion molecules, regeneration, neurodegenerative diseases

SUMMARY:

The combination of transmission electron microscopy and in utero transduction is a powerful approach for studying morphological changes in the fine ultrastructure of the nervous system during development. This combined method allows deep insights into changes in structural details underlying neuroplasticity with respect to their topographical representation.

ABSTRACT:

The present study combines in utero transduction with transmission electron microscopy (TEM) aiming at a precise morphometrical analysis of ultrastructural parameters in unambiguously identified topographical structures, affected by a protein of interest that is introduced into the organism via viral transfer. This combined approach allows for a smooth transition from macrostructural to ultrastructural identification by following topographical navigation maps in a tissue atlas. High-resolution electron microscopy of the in-utero-transduced tissue reveals the fine ultrastructure of the neuropil and its plasticity parameters, such as cross-sectioned synaptic bouton areas, the number of synaptic vesicles and mitochondria within a bouton profile, the

length of synaptic contacts, cross-sectioned axonal areas, the thickness of myelin sheaths, the number of myelin lamellae, and cross-sectioned areas of mitochondria profiles. The analysis of these parameters reveals essential insights into changes of ultrastructural plasticity in the areas of the nervous system that are affected by the viral transfer of the genetic construct. This combined method can not only be used for studying the direct effect of genetically engineered biomolecules and/or drugs on neuronal plasticity but also opens the possibility to study the in utero rescue of neuronal plasticity (e.g., in the context of neurodegenerative diseases).

INTRODUCTION:

No photon can penetrate an ultrathin tissue specimen in the depth grade of an electron. This attributes invaluable advantages to TEM in capturing nanometer resolution images of fine structures when compared to light microscopy techniques. For example, TEM allows for the visualization of intracellular organelles such as mitochondria, melanosomes, and various types of secretory granules, microtubules, microfilaments, cilia, microvilli, and intercellular junctions (cell surface specializations), in particular synapses in the nervous system[1–4]. The overall goal of the present methodological study is the ultrastructural recognition of changes in neural plasticity during development upon prenatal interference by combining the state-of-the-art techniques of in utero transduction and TEM. Virally encoded proteins of interest have been transduced in utero into the central nervous system[5–7], including the spinal cord[6]. For instance, in utero transduction in combination with TEM has been used for studying the effect of the cell adhesion molecule L1 on motor learning plasticity in L1-deficient mice, in particular with regard to the interplay between L1 and nuclear receptor proteins in cerebellar neurons[7].

The analysis of neuroplasticity parameters requires precise information about the localization of the smallest areas within the nervous system. Therefore, it is adequate to describe ultrastructural details and their exact topographical orientation with respect to other structures. In the present study, a specific preparatory method aiming at the detailed investigation of distinct morphological areas based on both light and electron microscopy is presented. This approach combines several techniques of tissue manipulation, starting with in utero transduction of the mouse brain and spinal cord and followed by perfusion fixation, mold-embedding, and processing the tissue for TEM. An essential step included between the embedding and the processing of the tissue for TEM is the documentation of the tissue, using the interference light reflection technique that allows for the precise microphotographic and low-magnification documentation of tissue specimens[8–10]. Incorporated into the present approach, this technique enables researchers to examine topographical and structural details of nervous tissue surfaces and of specimen slice profiles prior to their preparation for TEM.

A special frame for sectioning whole brains corresponds to stereotaxic coordinates. This frame benefits the morphological three-dimensional (3D) reconstruction of areas in nervous tissue and can be used for morphometric analysis. The macrographs of the visualized sections are assigned topographical coordinates, and the serially numbered sections build maps in a tissue atlas.

After resin processing, the embedded tissue is sectioned into ultrathin sections (<70 nm) containing selected areas, according to the maps of the above-mentioned tissue atlas. The

ultrathin sections are subjected to TEM to obtain high-resolution images of plasticity parameters (e.g., cross-section profile areas of synaptic boutons or axonal fibers) of their contents and of contacts to neighboring structures within the complex neuropil.

With the method described herein, the smooth transition from visualized macrostructures to micro- and nanostructures permits comparative in-depth studies of morphological neuronal plasticity after *in utero* transduction of the developing nervous system.

PROTOCOL:

All procedures on animal subjects have been approved by the institutional animal ethics committees of the federal states of Hamburg and Nordrhein-Westfalen, Germany.

1. In utero transduction

1.1. Prepare adeno-associated virus type 1 (AAV1) coding for the desired target (4 x 10^{11} viral particles/µL of AAV1) in phosphate-buffered saline (PBS) at pH 7.4. Add 0.1 mg/µL Fast Green and keep the AAV1-Fast-Green mixture at 37 °C.

1.2. Prepare a thin capillary tip with the desired shape (8 mm in length, with an outside diameter of 80 µm and an inside diameter of 50 µm), using a micropipette puller (settings: pressure = 500, heat = 700, pull = 0, velocity = 80, time = 200, see the **Table of Materials**). Break the tip of the capillary so that it is 4–5 mm.

1.3. Assemble an aspirator tube (44 cm x 0.7 cm) with the capillary tip and aspirate 15 µL of the AAV1-Fast-Green mixture into the capillary.

1.4. Keep the animal subjects at a constant physiological body temperature of 37 °C throughout the entire procedure.

1.5. Place a pregnant C57Bl/6 mouse (embryonic day 14.5) into the preincubation chamber and anesthetize the mouse with gaseous 4% isoflurane (with a volumetric airflow rate of 0.6–0.8 L/min).

1.6. Subcutaneously, inject buprenorphine (0.1 mg/kg of body weight).

1.7. Place the anesthetized mouse on the prewarmed surgical plate (37 °C).

1.8. Cover the eyes with a lubricant.

1.9. Fit the mouse with the anesthesia mask (gaseous 1.5 % isoflurane at a volumetric airflow rate of 0.6–0.8 L/min)on the surgical plate and shave the abdominal skin region. Wipe the shaved region with 75% ethanol.

NOTE: Monitor the breathing behavior of the anesthetized mouse continuously. Adjust the

concentration of the isoflurane gas according to the inhalation-exhalation pattern of the mouse.

1.10. Check for the absence of the plantar reflex by squeezing the hind paw phalanges of the mouse.

1.11. Open the abdominal cavity by gripping the skin with curved serrated iris forceps (10 cm) and cutting the skin along the *linea mediana* with straight tungsten carbide scissors (10 cm), and then, by gripping the peritoneal wall with straight Dumont tweezers (12 cm, 0.2 mm x 0.12 mm) and cutting the wall along the *linea alba* with straight Vanna's scissors (8 cm).

1.12. Place a piece of fenestrated paraffin film on the abdominal opening and fix the film on both ends with micro-mosquito hemostatic forceps (12.5 cm, curved).

1.13. Expose the uterine horns with a spoon-like device to avoid damage to the embryos inside the uterine horns. Drip a few drops of PBS (37 °C) on the uterine horns and inspect the embryos for damages or malformations inside the uterine sac.

1.14. Document the order and position of the embryos in the uterine horns. Turn the embryos carefully inside the uterine sac until the desired position for injection is reached.

1.15. Inject 1–2 µL of the AAV1-Fast-Green mixture by visually inspecting the injection site (e.g., brain ventricles) and the dye penetration under a stereomicroscope.

1.16. Document the injected embryos and place the uterine horns with the injected embryos back into the abdominal cavity.

1.17. Drip a few drops of PBS (37 °C) into the abdominal cavity. Close the cavity by suturing the peritoneal wall (use polyamide 6-0-sized sutures) and the skin (use polyamide 3-0-sized sutures), using Halsted's mosquito hemostatic forceps (12.5 cm, curved).

2. Telemacrophotography of isolated tissues

2.1. Preparation of buffers

2.1.1. Prepare **Sörensen's** buffer (1 L) by dissolving 14.95 g of Na_2HPO_4 and 2.18 g of KH_2PO_4 in 1 L of distilled water under stirring at 200 rpm.

2.1.2. Prepare **Mugnaini's** fixation solution (5 L) by heating 500 mL of distilled water to 75 °C and adding 50 g of paraformaldehyde powder under stirring at 200 rpm, adding 200 µL of 5 N NaOH, adding 1,500 mL of Sörensen's buffer, 1,750 mL of distilled water, and 500 mL of 25% glutaraldehyde. Fill up to 5,000 mL with distilled water. Use this final buffer for perfusion.

NOTE: Prepare Mugnaini's fixation solution under the hood, wear protective glasses, and avoid fumes. Add methylene blue (0.05 g/L) for a better visualization of the perfusion.

2.2. Mouse perfusion and tissue isolation

2.2.1. Transcardially perfuse the pregnant mice that carry the transduced embryos (in the case of embryo studies) or the born transduced pubs at the desired age (e.g., postnatal day 24) according to standard procedures[6,7,11–17], using intraperitoneal terminal sodium pentobarbital anesthesia (200 mg/kg of body weight).

2.2.2. Inject the mice transcardially with heparin solution (500 units) using a 26 G, 1 in needle and, before fixation, infuse the mice transcardially with 10 mL of PBS to flush out the blood from the body, and perfuse them transcardially with 30 mL of 40 °C prewarmed Mugnaini's fixation solution.

NOTE: For adult mice, perform an alternative retrograde perfusion via the abdominal aorta[14].

2.2.3. Isolate the perfused tissue of interest (e.g., whole brain or spinal cord) and postfix the tissue in at least 10 mL of Mugnaini's fixation solution for another 24 h at 4 °C.

2.2.4. Wash the tissue in 10 mL of PBS for 3 h at room temperature.

2.3. Embedding in agarose, plus documentation and sectioning

2.3.1. Adjust the isolated tissue (e.g., whole brain) in a special frame with a reproducible sectioning angle[8–10]. Alternatively, use a vibratome with an adjustable cutting thickness.

2.3.2. Place the nervous tissue in the frame, adjust the tissue for telemacrography, and document the coordinates.

2.3.3. Prepare 3% low-melting agarose-embedding medium: add 3 g of agarose in 100 mL of Sörensen's buffer and heat the mixture in a water bath to 90 °C.

2.3.4. Pour 3% agarose (30 °C) in the frame that contains the tissue. Cover the frame with a warm metal block and wait until it is hardening. During hardening, use telemacrographic devices to image the embedded tissue and its coordinates within the frame.

2.3.5. Transfer the agarose-embedded tissue into a frame with cutting gaps corresponding to the coordinates of the first frame.

2.3.6. Cut the embedded tissue into sections of desired thickness (e.g., 1.5 mm) with a device with a thin and vibrating razor blade (see **Table of Materials**).

NOTE: To improve the gliding of the razor blade, drip a few drops of glycerin onto the embedded tissue.

2.3.7. Image each tissue section in PBS and collect the images into a folder.

3. Preparation of the isolated tissue for transmission electron microscopy

NOTE: Perform all further steps of incubation in glass dishes with tightly closable lids on a shaking platform under the hood.

3.1. Wash the tissue sections for 2x 30 min in PBS. Incubate the sections in 2% aqueous osmium tetroxide solution (OsO_4) for 2 h at room temperature.

CAUTION: Osmium tetroxide is toxic and may be harmful when it comes in contact with skin.

3.2. Wash the osmicated sections for 2x 30 min in PBS.

3.3. Incubate the sections in 30%, 50%, and 70% ethanol at room temperature for 10–15 min (optional: incubate in 70% ethanol at 4 °C overnight).

3.4. Image the osmicated specimens in 70% ethanol under LED RGB light[8-10] (2x 15 W) applied to the sample from the left and right side at an angle of 45°. Use black dishes and a dull black background to minimize scattering and the reflection of the light during illumination.

CAUTION: Do not allow the section to dry out during imaging.

3.5. Create an atlas of the section images with coordinates by collecting images in series in a folder.

3.6. Incubate the specimens in 100% ethanol (2x for 30 min) and 100% propylene oxide (2x for 30 min) at room temperature.

CAUTION: Do not allow the sections to dry out while changing solutions.

3.7. Mix 260 mL of resin with 240 mL of dodecenylsuccinic anhydride in a glass vessel while gently stirring with a glass bar. Periodically check for inhomogeneity, bubbles, and smears. Very gently, stir by hand for at least 45 min.

3.8. Prepare resin/propylene oxide in a ratio of 1:2 and 1:1 and add 3% accelerator (2,4,6-Tris(dimethylaminomethyl)phenol).

3.9. Incubate the tissue in the 1:2 embedding solution from step 3.8 for 2 h and then in the 1:1 embedding solution from step 3.8 for 2 h at room temperature on a rotating wheel.

3.10. Place the tissue in flat polypropylene dishes, cover the tissue with fresh resin containing 3% accelerator, and cure the embedded tissue at 65–85 °C for 12–24 h.

3.11. Cool down the embedded tissue to room temperature and remove the resin-embedded specimens from the polypropylene dishes.

4. Selection of ultrastructural neuroplasticity parameters for quantitative analysis

4.1. Mapping the area of interest

4.1.1. Choose an area of interest (e.g., hippocampus or cerebellum) and localize the area in the section atlas by choosing the image from the atlas (step 3.5) that contains this area.

4.1.2. Sketch the borders of the area of interest onto the section image and find/superimpose these region borders onto the resin specimen.

4.1.3. Scratch-mark the borders of the area of interest (e.g., hippocampus or cerebellum) on the resin specimen, using a fine needle gauge (26 G, 1 in).

4.1.4. Heat the resin specimen to 85 °C in an oven to soften the resin for trimming or, alternatively, use a trimming device, a thin blade, or sandpaper.

4.1.5. Excise the area of interest from the resin specimen with a razor blade (see **Table of Materials**). Mount the specimen on holding bars of acrylic glass of the required caliber (e.g., with a diameter of 8 mm and a length of 1 cm) with glue. Trim the mounted specimen for semi- and ultrathin sectioning.

4.1.6. Prepare semithin (0.75 µm) and ultrathin (70 nm) sections of the trimmed area using an ultramicrotome: set it at 1.5 mm/s for 0.75 µm thickness and at 0.7 mm/s for 70 nm thickness.

4.1.7. Collect the semithin sections on glass carriers and stain the sections with 1% toluidine blue in PBS (for 4 min).

4.1.8. Wash the sections several times in deionized water. Examine the stained sections under the light microscope using 4x (NA of 0.1 ∞/-), 10x (NA of 0.22 ∞/0.17), 40x (NA of 0.65 ∞/0.17), and 100x (NA of 1.25 ∞/0.17) objectives.

4.1.9. Collect ultrathin sections on nickel grids. Subject the grids to TEM at 180 kV and at 3,200x, 6,000x, and/or 8,000x magnification.

4.2. TEM Analysis

4.2.1. Choose the ultrastructural parameters of interest for quantitative TEM analysis (e.g., boutons with vesicles and mitochondria or myelinated and nonmyelinated axons) and take TEM images of these parameters under 3,500x, 6,000x and/or 8,000x magnification.

REPRESENTATIVE RESULTS:

For reliable and fast anesthesia of mice, numerous safety parameters were considered, and an optimized workspace of the anesthesia unit proved to be adequate (**Figure 1A**). The unit is designed to control the mixture of liquid isoflurane and ambient air with a precision required for successful surgery on small animals, such as mice and rats. Air and isoflurane are mixed in the vaporizer according to the desired settings and delivered into a box or through a mask to the animal (**Figure 1A**). The scavenger collects and inactivates any surplus isoflurane gas that may be produced, thus providing a safe working environment. The gas is collected and passed through active coal in a cartridge (**Figure 1A**).

An optimized set of instruments (**Figure 1B**) allows scientists to perform surgery quickly on pregnant mice. A simple paraffin film with hydrophobic properties prevents the abdominal mucosa from drying after resection (**Figure 1B**). The uterine horns harboring the embryos are exposed onto paraffin film and the embryos are numbered in the way shown in **Figure 1C**. Once localized, the embryos are adjusted in the correct position for injection of the virus via a thin capillary (**Figure 1D**). The injection is visually controlled by observing the diffusion shape of the penetrating dye contained in the injected virus mixture (**Figure 1D**). After in utero injection, the uterine horns are placed back in the abdominal cavity to allow the development of the injected embryos until the stage (e.g., until birth and reaching postnatal day 24) that is needed for the experiment (see, for example, Lutz et al.[5,6] and Kraus et al.[7]). For studies on synaptic plasticity, late developmental or adult stages are recommended.

After a transcardial perfusion of the animals at the desired stage, the nervous tissue (for example, the brain and spinal cord in **Figure 2**) is placed and oriented in a transparent plastic frame with coordinates and embedded in agarose (**Figure 2A,B**). After cutting, every section is imaged (**Figure 2C,E,G**) and then osmicated. After osmication and incubation in 70% or 80% ethanol, the sections are imaged again by using interference light telemacrography[8–10]. The tissue sections must be placed in a dull black surrounding to minimize scattered radiation during the required high-intensity illumination. Fiber tracts and different areas of the tissue reflect iridescent colors that contrast the dark tissue background, and a specifically colored pattern of the tissue surface emerges (**Figure 2D,F,H**). All iridescent images are superimposed with the nonosmicated images and, together with the projected coordinates of the embedding step, they generate navigation maps of the tissue topography. Next, the tissue is further dehydrated and embedded into resin. Based on the navigation maps, areas of interest are chosen and further processed for TEM—as examples, the hippocampus (*stratum lucidum*), cerebellum (granule cell layer), and spinal cord (dorsal funiculus) are shown in **Figure 3A1–C4**.

In the hippocampus and cerebellum, mossy fiber synapses are known to exhibit unique ultrastructural characteristics when compared to other synapses, including large presynaptic boutons (**Figure 3A1–B5**). They can be easily localized with the help of the iridescent navigation maps established during the previous processing steps. In their cross-section profiles, the boutons contain a vast number of vesicles and mitochondria and very often enclose several dendritic spines (**Figure 3A4,A5,B4,B5**). TEM images of these profiles allow a quantification of cross-sectioned synaptic bouton areas, numbers of synaptic vesicles and mitochondria within a bouton profile, the length of synaptic contacts, and numbers of contact sites.

In the spinal cord, the dorsal funiculus contains many axonal fibers which are myelinated by oligodendroglia. On transversal sections, the dorsal funiculus can be found between both posterior horns of the spinal cord on transversal sections (**Figure 3C1,C2**). On TEM images, cross-sectioned myelinated axons appear round and the myelin sheaths surrounding the axons are organized in lamellae (**Figure 3C3,C4**). A quantification of cross-sectioned axonal areas, including the thickness of myelin sheaths and the number of myelin lamellae, as well as a quantification of cross-sectioned mitochondria profile areas can be carried out.

The prepared atlas and the micrographic TEM images of the ultrastructural parameters are not only helpful for a comparison of morphological neuroplasticity upon different conditions of treatment, but also when a comparison between parameters within the same area is required (e.g., a comparison between different type of synapses in the hippocampus[18] and between equivalent structures of different species [**Figure 4**]).

FIGURE AND TABLE LEGENDS:

Figure 1: Preparation for in utero transduction. (**A**) Isoflurane anesthesia setup: 1) induction chamber for initial anesthesia; 2) pump; 3) anesthesia unit; 4) routing switch valve; 5) anesthesia supply tubing; 6) scavenger unit; 7) breathing mask; 8) heated surgical table; 9) binocular stereoscope with light source; 10) control unit. (**B**) Surgery equipment: 1) electric shaver; 2) eye lubricant; 3) fixation tapes; 4) sanitary pads; 5) iris forceps (10 cm, curved, serrated); 6 and 7) iris scissors (11 cm, straight, tungsten carbide); 8) Dumont tweezers (#3, 12 cm, straight, 0.2 mm x 0.12 mm); 9) Vanna's scissors (8 cm, straight); 10 and 11) micro-mosquito hemostatic forceps (12.5 cm, curved); 12) paraffin film; 13) micro spoon; 14) cotton swabs; 15 and 16) Halsted's mosquito hemostatic forceps (12.5 cm, straight); 17 and 18) sutures (size 6-0 and 3-0); 19) syringe; 20) aspirator tube assemblies for calibrated microcapillary pipettes. **(C)** Scheme of a systematic numbering of embryos within the uterine horns at embryonic day (E) 14.5: L = left; R = right. **(D)** Strategies of injection: into the first and second brain ventricles (I^{ve} and II^{ve}, respectively), fourth ventricle (IV^{ve}), and spinal cord.

Figure 2: Documentation of isolated tissue by telemacrography. (**A** and **B**) Embedding of a brain and a spinal cord in agarose, using cuvettes with a coordinate system (grids). After cutting, the sections are subjected to telemacrography and interference reflection light imaging. (**C**–**F**) Transversal and sagittal sections of the brain. The scale bars = 5 mm. co = cortex; st = striatum; di = diencephalon; me = mesencephalon; hi = hippocampus; ped = *pedunculi cerebri*; ce = cerebellum; mo = *medulla oblongata*. (**G** and **H**) Transversal section of a cervical segment of the spinal cord. The scale bar = 1 mm. ahR and ahL = right and left anterior horn, respectively; df = dorsal funiculus.

Figure 3: Topographical and ultrastructural identification of plasticity parameters. (**A1**) Inverted interference light macrograph of the hippocampus. The region of the mossy fiber boutons within the *stratum lucidum* (sl) is highlighted. pcl = pyramidal cell layer; gcl = granule cell layer. The scale bar = 500 µm. (**A2**) Light microphotograph (100x objective) of a semithin section (0.75 µm)

showing *stratum lucidum*. The asterisks indicate the regions of mossy fiber boutons that surround many dendritic islands (black arrowheads). Toluidine blue/OsO_4 staining. The scale bar = 50 µm. (**A3**) Transmission electron micrograph at a low magnification, showing mossy fiber boutons (asterisks) surrounding a dendrite (D). The rectangle includes a single mossy fiber bouton. The scale bar = 2 µm. (**A4**) Transmission electron micrograph of the cross-section profile of a single mossy fiber bouton (MFB). Spines (blue) and the cross-section bouton area (violet) are highlighted. m = mitochondrion; s = spines. The scale bar = 200 nm. (**A5**) Synaptic vesicles inside an MFB. The scale bar = 100 nm. (**B1**) Inverted interference light macrograph of the cerebellum. The region of the granule cell layer (gcl) that contains the mossy fiber boutons is indicated. mcl = molecular layer; h = hilus. The scale bar = 500 µm. (**B2**) Light microphotograph (100x objective) of a semithin section (0.75 µm) showing a region of the granule cell layer that borders on Purkinje cells (PC). The asterisks indicate the regions of mossy fiber boutons that are surrounded by many cerebellar granule neurons (CGN). Toluidine blue/OsO_4 staining. The scale bar = 50 µm. (**B3**) Transmission electron micrograph at a low magnification, showing the area of MFBs. The rectangle includes a single MFB. The scale bar = 2 µm. (**B4**) Transmission electron micrograph of the cross-section profile of a mushroom-like MFB within the granule cell layer of the cerebellum. The cross-section bouton area (violet) and vesicle-free spines (blue) are highlighted. The scale bar = 1 µm. (**B5**) Mitochondria (violet) and synaptic vesicles inside an MFB. The scale bar = 150 nm. (**C1**) Inverted interference light macrograph of the spinal cord. The region of the dorsal funiculus (df) that contains heavily myelinated axons is highlighted. ahR and ahL = right and left anterior horn, respectively; phR and phL = right and left posterior horn, respectively. The scale bar = 500 µm. (**C2**) Light microphotograph (100x objective) of a semithin section (0.75 µm) showing the dorsal funiculus that is enriched with heavily myelinated axons (ax) that are clearly distinguishable from nonmyelinated fibers (asterisks). ct = connective tissue. Toluidine blue/OsO_4 staining. The scale bar = 50 µm. (**C3**) Transmission electron micrograph of cross-section profiles of myelinated and nonmyelinated axons (ax, violet). my = myelin sheaths. The scale bar = 200 nm. (**C4**) The rectangle shows high-magnified, heavily myelinated axons with myelin lamellae and nonmyelinated fibers. The microtubules (mt) and mitochondria (m) within the axonal fibers are visible. The scale bar = 50 nm.

Figure 4: Examples of ultrastructural plasticity parameters in different animal models. (**A**) Mossy fiber boutons in the hippocampus of a shark. The scale bar = 400 nm. (**B**) Mossy fiber bouton in the hippocampus of a rhesus macaque. The scale bar = 200 nm. (**C**) Neuromuscular junction of a cat. The scale bar = 300 nm. (**D**) Synaptic boutons in the optical tectum of a phalanger. The asterisks indicate postsynaptic density (PSD; in inset: black arrowheads). The rectangle shows a magnified PSD. The scale bar = 400 nm. (**E**) A nonmyelinating Schwann cell (SC) surrounding many axonal fibers in the spinal ganglion of a mouse. In the vicinity, heavily myelinated fibers are visible. The scale bar = 300 nm. For all panels: ax = axon; D = dendrite; m = mitochondrion; MFB = mossy fiber bouton; MF = mossy fiber; my = myelin sheaths; s = spine; sa = spine apparatus; sv = synaptic vesicles. Contact sites (postsynaptic density) are indicated with asterisks. Cross-section areas are highlighted in violet and blue.

DISCUSSION:

A crucial step of in utero transduction is the injection procedure. The precise injection into brain

ventricles or into another area of interest requires experience and hands-on skill. The thinner the microcapillary tip, the less tissue damage may occur; however, this is at the cost of increasing injection pressure. In contrast to in utero electroporation[19–22], the survival rate of the injected embryos after in utero transduction is very high. All embryos of the uterine horn can be injected, even if embryos are located at the roots of the uterine horns and need to be readjusted within the uterine sac. The developmental stages of injection and analysis can be adapted to the scientific question.

When injected into the brain ventricles, the AAV1 particles are distributed via the cerebrospinal fluid through the entire ventricular system. Developing structures with surfaces that are in close contact with the liquor, such as the hippocampus and the cerebellum, are transduced by the applied viral particles[5–7]. An analysis of the changes in ultrastructural parameters, such as bouton size, cross-sectioned bouton areas, numbers of synaptic vesicles, numbers of mitochondria, and numbers and length of synaptic contacts in these structures, is an elegant read-out for ultrastructural plasticity. The in utero transduction method can be applied to study different brain areas, as well as the spinal cord[6]. Moreover, other organs and tissues can similarly be chosen as transduction targets. Finally, the frequently used mouse model can be replaced by other species as required.

The photography of the tissue slices allows for a very precise dissection of resin-embedded samples for transmission electron microscopy. Since the spatial orientation of the sample is defined, extensive and time-consuming cutting was omitted. Moreover, crucial neighboring regions can be kept in their original relationship to each other. An important advantage of the photographic documentation step is the possibility to monitor the quality of tissue fixation and to recognize pathological alterations. Thus, the quality of fixation and embedding of the material is of pivotal importance. Nonsuitable samples that contain artifacts can be outsourced prior to further processing.

The micrograph series of the imaged tissue slices provide a helpful morphological atlas. For comparative neuroanatomical studies, which may also include rare species, the atlas is a valuable archive. The choice of thickness of the tissue slices depends on the addressed scientific question and is limited by the size of the tissue specimen. For small animals, a thickness of up to 1 mm should be adjusted. Very thick sections (>5 mm) are not recommended, due to the limited penetration capacity of osmium tetroxide[8–10].

The telemacrophotography documentation is essential for morphometrical studies. It offers a fixed correlation of the tissue surface to the side and top plane of the frame and defines the position of a given structure in a spatial system of coordinates. A combination of the described method with electrophysiology[18] or with the spinal cord injury model, where plasticity plays an essential role in regeneration[6], is possible. For comparative neuroanatomical studies, which may also include rare species, the iridescent tissue atlas and the micrographic TEM images of the ultrastructural parameters are valuable assets.

Despite its strengths, TEM has several limitations due to the thickness size of the sample (70 nm),

the cross-sectioned area (<6 mm^2), and the complexity of the sample preparation. However, when a specimen is accurately prepared, the TEM images of the specimen's ultrastructure are of superior quality in comparison to images produced by any light-microscopical techniques. The in utero transduction technique is limited by the injected volume and the topographical region of the injection. Indisputably, the technique has a strong translational medical potential in early developmental treatment and in the rescue of congenital neuropathological diseases. Moreover, the present combination of in utero transduction with TEM might not only be considered as an approach for the future treatment of congenital diseases, but also for high-quality ultrastructure-based neurodevelopmental diagnostics.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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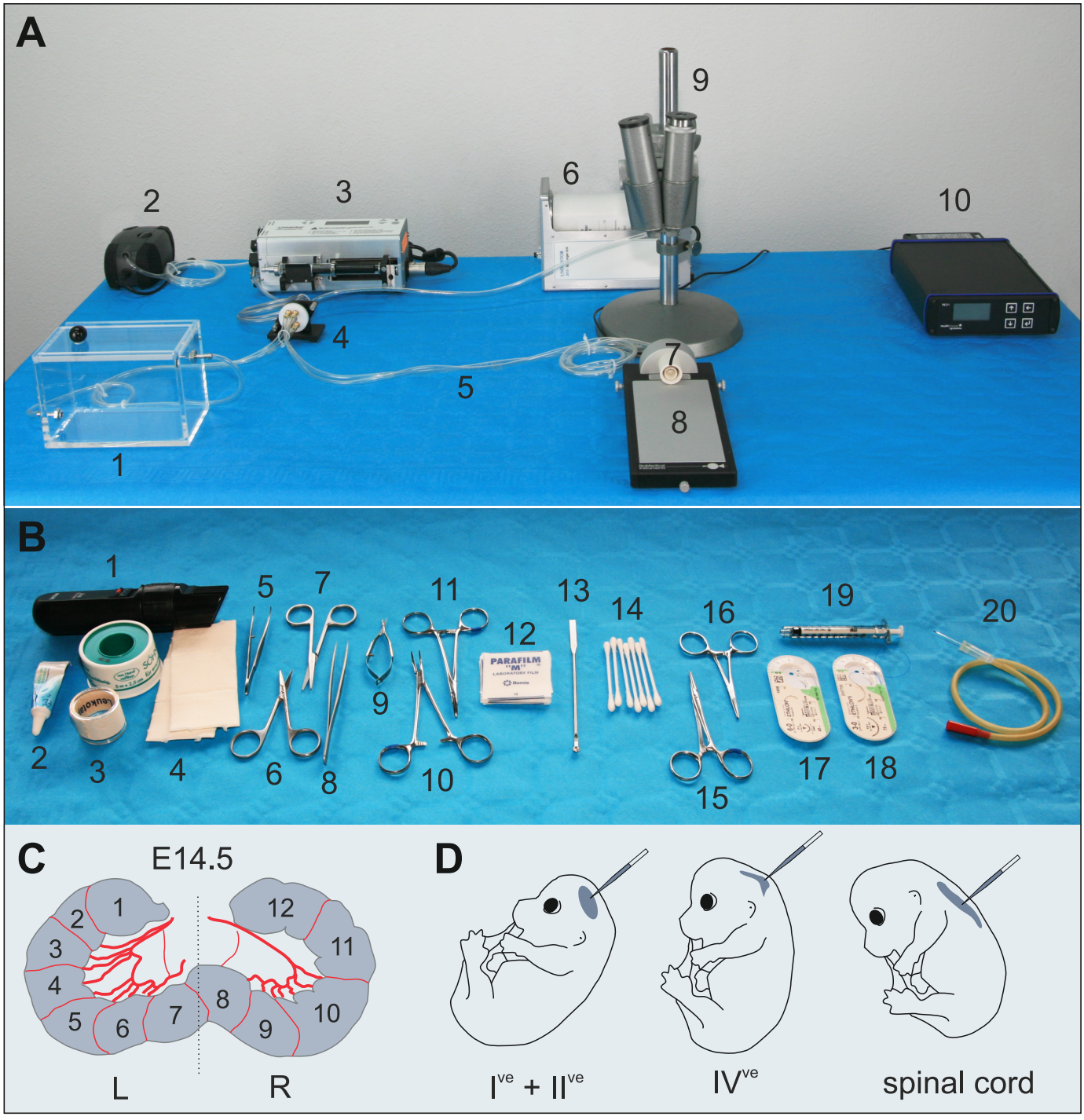

Fig. 1

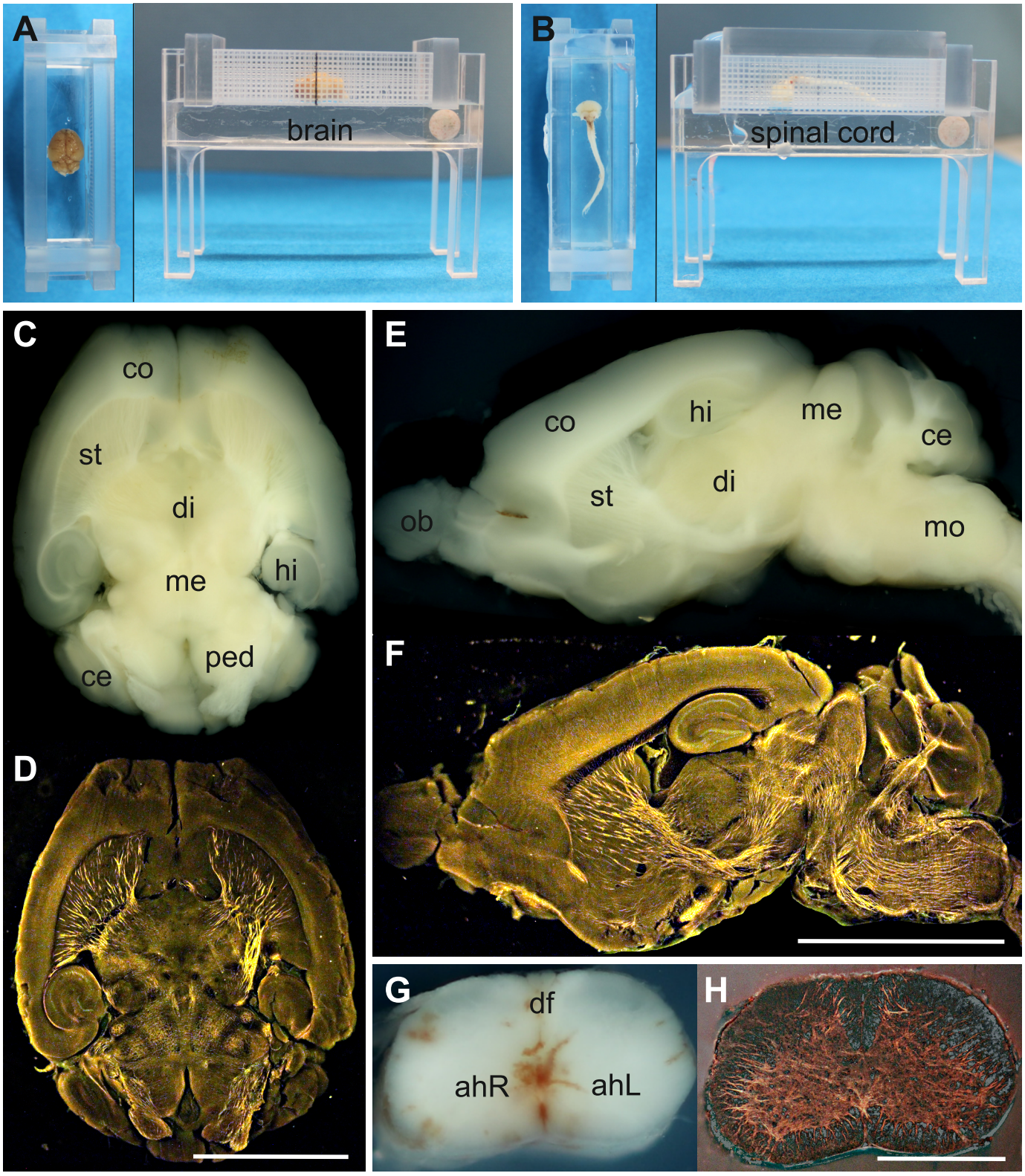

Fig. 2

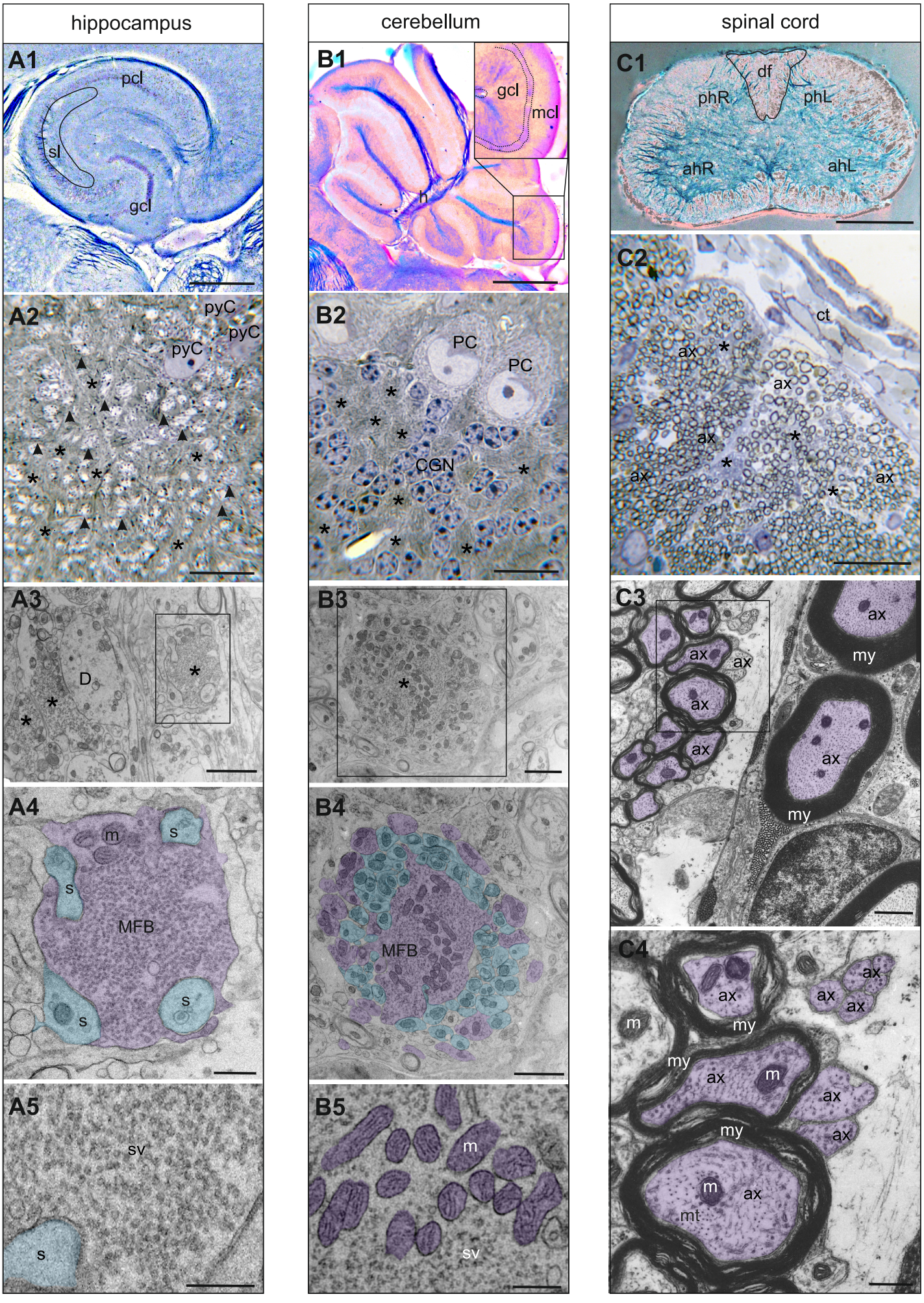

Fig. 3

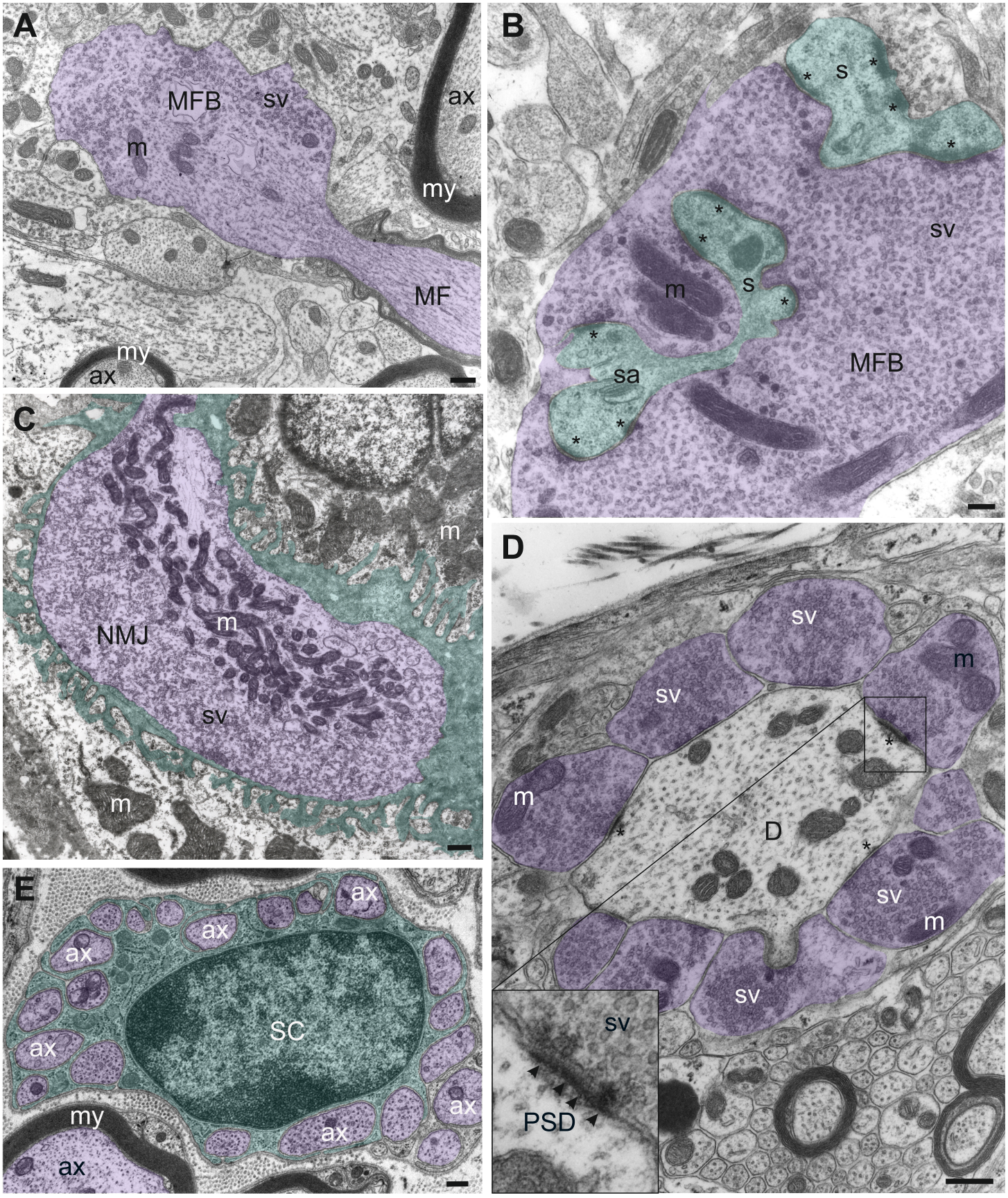

Fig. 4

Table of Materials

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2,4,6-Tris(dimethyl-aminomethyl)phenol	Serva	36975	
26Gx 1'' needle	Henke-Sass, Wolf GmbH		
410 Anaesthesia Unit for air pump	Biomedical Instruments (Univentor)	8323102	
Adeno-associated virus serotype 1 (AAV1)	UKE (Viral Core Facility)	-	For references and target areas of AAV1 see: https://www.addgene.org/viral-vectors/aav/aav-guide/ and also: Designer gene delivery vectors: molecular engineering and evolution of adeno-associated viral vectors for enhanced gene transfer. Kwon I, Schaffer DV. Pharm Res. 2008 Mar;25(3):489-99. Recombinant AAV viral vectors pseudotyped with viral capsids from serotypes 1, 2, and 5 display differential efficiency and cell tropism after delivery to different regions of the central nervous system. Burger C, Gorbatyuk OS, Velardo MJ, Peden CS, Williams P, Zolotukhin S, Reier PJ, Mandel RJ, Muzyczka N. Mol. Ther. 2004 Aug;10(2):302-17. Self-complementary recombinant adeno-associated virus (scAAV) vectors promote efficient transduction independently of DNA synthesis. McCarty DM, Monahan PE, Samulski RJ. Gene Ther. 2001 Aug;8(16):1248-54.
Agarose	Sigma-Aldrich	A9414	low gelling agarose
Air Pump	Biomedical Instruments (Univentor)	Eheim 100	
Araldite	CIBA-GEIGY	23857.9	resin for embedding of tissue
aspirator tune assemblies	Sigma-Aldrich	A5177-5EA	
Breathing Mask Mouse Anodized Aluminium	Biomedical Instruments (Univentor)	-	
buprenorphine	Temgesic	ampules	painkiller
capillaries	Science-Products	GB100TF-10	with fillament
Dodecenylsuccinic anhydride	Fluka	44160	

Dumont tweezers (#3, 12 cm, straight, 0.2 x 0.12 mm)	FST	11203-23	
electric shaver	Phillips	-	
Ethicon sutures (Ethilon, 6-0 and 3-0)	Ethicon	-	polyamide
eye lubricant	Bepanthene	-	
Fast Green	Sigma-Aldrich	F7252	for visualization of injected liquids
Gas Routing Switch 4/2 connectors	Biomedical Instruments (Univentor)	8433020	
halsted Mosquito hemostatic forceps (12.5 cm, straight)	FST	13011-12	
Heparin-Natrium	Ratiopharm		25 000 I.E./5 ml
Induction box for mice with horizontally moving lid.	Biomedical Instruments (Univentor)	-	
iris forceps (10cm, curved, serrated)	FST	14007-14	
iris scissors (11cm, straight, tungsten carbide)	FST	14501-14	
Isofluran OP Tisch, electrically heated, sm Outer dimensions: 257x110x18 mm.	Biomedical Instruments (Univentor)	-	
isoflurane (Attane)	JD medical		inhalation anesthesia
LED RGB lights	Cameo	CLQS15RGBW	LEDs 2 x 15 W
Light microscope Basic DM E	Leica	-	4x (N.A. 0.1 ∞/-), 10x (N.A. 0.22 ∞/0.17), 40x (N.A. 0.65 ∞/0.17), 100x (N.A. 1.25 ∞/0.17) objectives
micropipette puller	Science-Products	P-97	

Mosquito hemostatic forceps (12.5cm, curved)	FST	13010-12	
Nickel grids, 200 mesh	Ted Pella	1GC200	
Osmium (VIII)-oxid	Degussa	73219	
Propylene oxide	Fluka	82320	
razor blades	Schick	87-10489	
Sodium pentobarbital (Narcoren)	Merial GmbH	-	
TC01mR 1-Channal temperature controller wit	Biomedical Instruments (Univentor)	-	
Technovit 4004 two components glue	Kulzer		
Telemacrodevice	Canon	-	Canon Spiegelreflex Kamera EOS2000D, EF-S 18-55 mm f/3.5-5.6 IS STM Objective, Extension below 150 mm, Manual Extension Tube 7 mm ring, 14 mm ring, 28 mm ring, Macro reverse ring (58 mm), Canon copy stand.
Thermopuller P-97	Sutter Instruments	-	
thin vibrating razor blade device	Krup	-	with Szabo thin blades
toluidine blue	Sigma-Aldrich	89640	
Transmission electron microscope C20	Phillips	-	up to 200 kV
Tygon 6/4 Tubing material for connection of all parts Outer diameter: 6mm Inner diameter: 4mm Wa ll thickness: 1mm	Biomedical Instruments (Univentor)	-	
Ultracut E	Reichert-Jung	-	ultramicrotome
Univentor Scavenger	Biomedical Instruments (Univentor)	8338001	
Vannas scissors (8 cm, straight)	FST	15009-08	

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Author(s): DAVID LUTZ, MONIKA VON DÜRING, FRANCO CORVACE, LUZIE AUGUSTINOWSKI, ANNE-KATHRIN TRAMPE, MARZENA NOWAK, ECKART FÖRSTER

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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.