Video Article

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

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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[1,2,3,4]. 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[5,6,7], 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[8,9,10]. 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. 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.
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.
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.
4. Keep the animal subjects at a constant physiological body temperature of 37 °C throughout the entire procedure.
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).
6. Subcutaneously, inject buprenorphine (0.1 mg/kg of body weight).
7. Place the anesthetized mouse on the prewarmed surgical plate (37 °C).
8. Cover the eyes with a lubricant.
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.
10. Check for the absence of the plantar reflex by squeezing the hind paw phalanges of the mouse.
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).
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).
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.
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.
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.
16. Document the injected embryos and place the uterine horns with the injected embryos back into the abdominal cavity.
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

1. **Preparation of buffers**
   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. 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. **Mouse perfusion and tissue isolation**
   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[6,7,11,12,13,14,15,16,17], using intraperitoneal terminal sodium pentobarbital anesthesia (200 mg/kg of body weight).
   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].
   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.
   4. Wash the tissue in 10 mL of PBS for 3 h at room temperature.

3. **Embedding in agarose, plus documentation and sectioning**
   1. Adjust the isolated tissue (e.g., whole brain) in a special frame with a reproducible sectioning angle[8,9,10]. Alternatively, use a vibratome with an adjustable cutting thickness.

2. Place the nervous tissue in the frame, adjust the tissue for telemacrography, and document the coordinates.
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.
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.
5. Transfer the agarose-embedded tissue into a frame with cutting gaps corresponding to the coordinates of the first frame.
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.
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.

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.
2. Wash the osmicated sections for 2x 30 min in PBS.
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).
4. Image the osmicated specimens in 70% ethanol under LED RGB light[8,9,10] (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.
5. Create an atlas of the section images with coordinates by collecting images in series in a folder.
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.
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.
8. Prepare resin/propylene oxide in a ratio of 1:2 and 1:1 and add 3% accelerator (2,4,6-Tris(dimethylaminomethyl)phenol).
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.
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.
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

1. **Mapping the area of interest**
   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.
   2. Sketch the borders of the area of interest onto the section image and find/superimpose these region borders onto the resin specimen.
   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. 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.
   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.
   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.
   7. Collect the semithin sections on glass carriers and stain the sections with 1% toluidine blue in PBS (for 4 min).
   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.
   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.

2. **TEM Analysis**
   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[8,9,10]. 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**]).

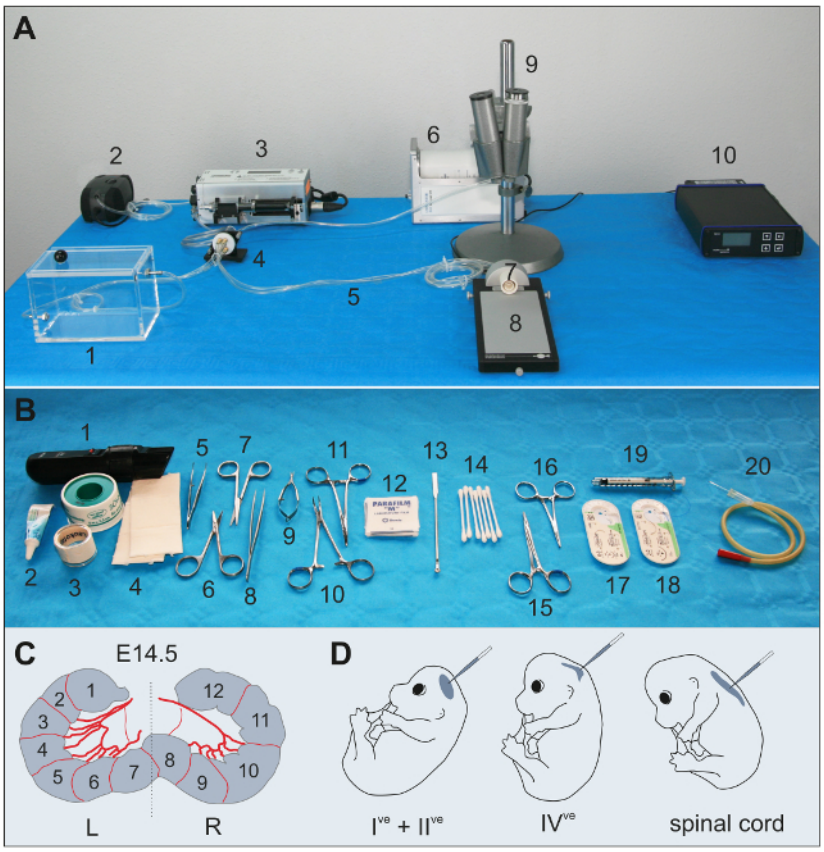

Figure 1: Preparation for in utero transduction. (**A**) Isoflurane anesthesia setup: 1) induction chamber for initial anesthesia; 2) pump; 3) anesthesia unit; 4) routing switch valve; 5) anesthesia supply tubing; 6) scavenger unit; 7) breathing mask; 8) heated surgical table; 9) binocular stereoscope 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. Please click here to view a larger version of this figure.

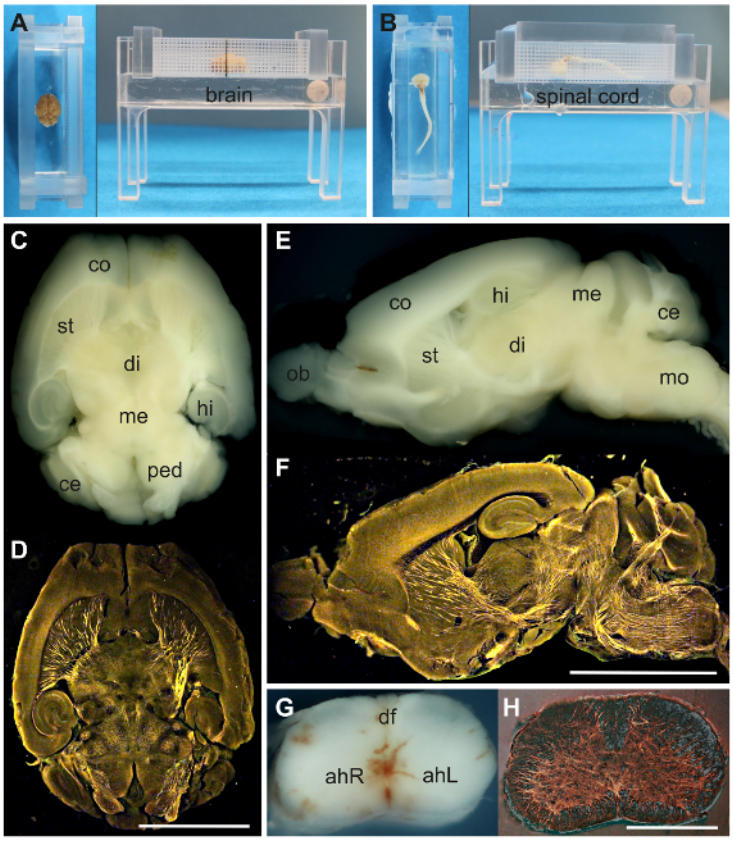

Figure 2: Documentation of isolated tissue by telemacrography. (**A** and **B**) Embedding of a brain and a spinal cord in agarose, using cuvettes with a coordinate system (grids). After cutting, the sections are subjected to telemacrography and interference reflection light imaging. (**C**-**F**) Transversal 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. Please click here to view a larger version of this figure.

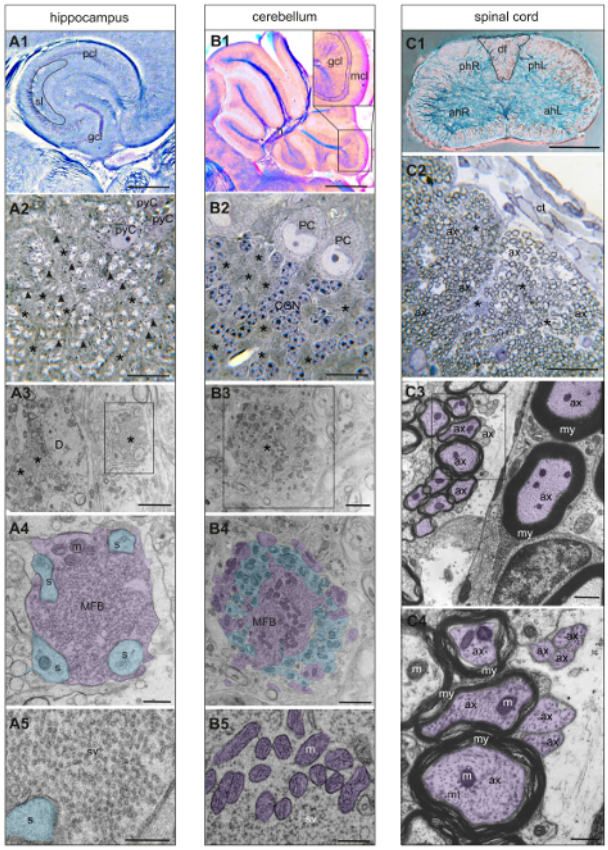

Figure 3: Topographical and ultrastructural identification of plasticity parameters. (**A1**) Inverted interference light macrograph of the hippocampus. The region of the mossy fiber boutons within the *stratum lucidum* (sl) is highlighted. pcl = pyramidal cell layer; gcl = granule cell layer. The scale 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. Please click here to view a larger version of this figure.

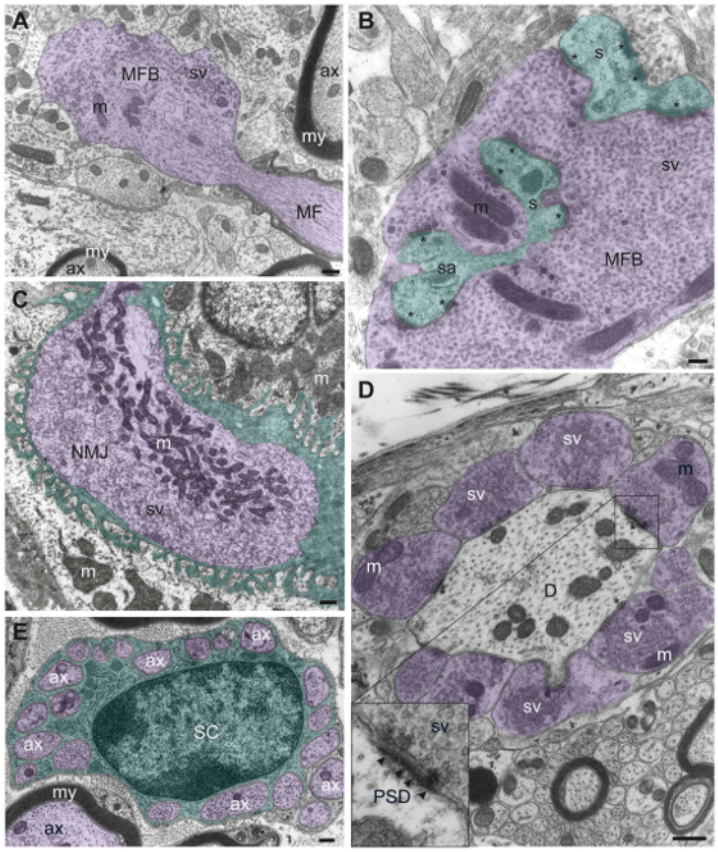

Figure 4: Examples of ultrastructural plasticity parameters in different animal models. (**A**) Mossy fiber boutons in the hippocampus of a shark. The scale bar = 400 nm. (**B**) Mossy fiber bouton in the hippocampus of a rhesus macaque. The scale bar = 200 nm. (**C**) Neuromuscular junction of a cat. 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. Please click here to view a larger version of this figure.

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[19,20,21,22], 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[5,6,7]. 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[8,9,10].

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.

Disclosures

The authors have nothing to disclose.

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