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Cortical Organotypic Brain Slice Cultures to Examine Sex- and Age-Dependent Astrocyte-Mediated Synaptic Phagocytosis

Ex vivo Examination of Astrocyte-Mediated Synaptic Phagocytosis

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Abstract: Astrocytes, the most abundant glial cells in the brain, are an integral part of the synaptic compartment and contribute to synaptic pruning, a key process for refining neural circuits during early postnatal development (PND). Dysregulations in this process are implicated in various neuropsychiatric disorders, including major depressive disorder (MDD). To investigate astrocyte functions in a physiologically relevant context, organotypic brain slice cultures (OBSCs) offer a powerful model, reproducing more closely in vivo conditions than traditional cell cultures and preserving complex brain architecture and interactions. Here, we present OBSCs as an ex vivo culturing method to provide a platform to explore astrocyte-mediated synaptic pruning dynamics in the rat prefrontal cortex (PFC) during PND. Our approach is based on assessing the role of MEGF10, a key protein involved in synaptic pruning, alongside the synaptic markers synaptophysin and PSD95, using Western blotting to analyse the expression levels of these markers in the cortex of developing rat pups. Additionally, we combine immunofluorescence staining with confocal imaging and IMARIS 9.8 software-assisted analysis to investigate the co-localization of the lysosomal marker LAMP1 with synaptic and astrocytic markers to evaluate the precise rate of synaptic engulfment. The methods presented here allow a deeper examination of an astrocyte-mediated synaptic remodelling in healthy and pathophysiological conditions.

Keywords: organotypic brain slice culture; astrocyte; phagocytosis; synaptic elimination; prefrontal cortex; critical period; major depressive disorder

1. Introduction

Astrocytes, the predominant subtype of glial cells in the central nervous system (CNS), present diverse functions crucial for brain homeostasis and function. They support endothelial cells at the blood-brain barrier (BBB), regulate ion homeostasis around synapses, provide metabolic support to neurons, and play a crucial role in synaptogenesis [1–3]. During the critical period of postnatal brain development in rodents, astrogenesis goes along with active synaptogenesis, leading to the formation of functional neuronal networks [4–6].

Altered properties of astrocytes have been shown in various neuropsychiatric disorders, including Alzheimer's disease [7], Parkinson's disease [8], epilepsy [9], and major depressive disorder (MDD) [10]. In particular, astrocytes play an important role in synaptic pruning, a process necessary for refining neural circuits during development [11]. The prefrontal cortex (PFC) is especially susceptible to environmental and endogenous influences during early postnatal development, affecting its structural and functional maturation and potentially shaping neural and behavioral long-term outcomes [12,13]. Astrocyte-mediated synapse elimination involves the activation of signalling systems such as the MEGF10 phagocytic pathway. MEGF10 is a protein predominantly expressed in astrocytes and is essential for maintaining synaptic homeostasis [14].

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While primary dissociated cultures facilitate the study of single-cell populations, organotypic brain slice cultures (OBSCs) offer a more comprehensive model to analyze interactions between various brain cell types. OBSCs preserve the structural and synaptic organization of the brain, making them valuable for ex vivo investigations of cellular and molecular processes [15–17]. Moreover, OBSCs present a more ethical and efficient alternative to in vivo studies, reducing the need for animal models.

In this chapter, we describe two approaches to investigate astrocyte-mediated phagocytosis of synapses in the rat PFC during postnatal development using OBSCs. Adapted from Stoppini, Buchs, and Muller (1991) [18] and Humpel (2018) [19], our methodology incorporates sensitive immunofluorescent-immunohistochemical staining to detect glial fibrillary associated protein (GFAP, an astrocytic marker), along with synaptophysin (a presynaptic marker) and lysosomal associated membrane protein 1 (LAMP1, a lysosomal marker). Additionally, this protocol includes Western blot analysis to characterize the expression of synaptic markers PSD95 and synaptophysin, alongside the phagocytic receptor MEGF10, in lysates from cortical tissue.

Together, these methods are helpful to get a better understanding of astrocytic mechanisms underlying brain disorders such as MDD, while preserving the complexity of the cellular environment and minimizing animal use.

2. Materials

2.1. Animals

1. Male and female non-selectively bred Wistar (NAB) or Wistar Kyoto (WKY) rats at between postnatal (P) days 4 to 7 were used (purchased from Charles River Laboratories). Procedures follow institutional guidelines of the University of Regensburg, Germany, as well as the European Community Council Directive (86/609/EEC). All experiments are approved by the Animal Welfare Office at the Animal Care and Use Facility of the University of Regensburg. In accordance with the German Animal Welfare Act (Tierschutzgesetz, Articles 4 and 7), no additional approval for the post-mortem removal of brain tissue is necessary. All efforts were taken to minimize animal pain or discomfort.

2.2. Preparation of Organotypic Brain Slice Cultures (OBSCs)

1. Dissection tools: surgical scissors (sharp-blunt, straight), dissection scissors (sharp), scalpel, standard pattern forceps (serrated), narrow pattern forceps (curved and straight), spatula (with microspoon).
2. 3.5 cm dissection dish.
3. Sterivex filters (0,22µm).
4. Dissection medium with $CaCl_2$ (1mM), D-glucose (10 mM), KCl (4mM), $MgCl_2$ (5mM), $NaHCO_3$ (26mM), Sucrose (234mM), Phenol Red (0,1%), penicillin–streptomycin (100 U/mL).
5. OBSC culture medium with MEM (Minimum Essential Medium), EBSS (Earle's balanced salt solution), 25 mM HEPES, 25% heat-inactivated horse Serum, 5 mg/mL D-glucose, 1 mM GlutaMAX, 1% penicillin–streptomycin.
6. 6-well plates.
7. 30mm culture plate inserts (0,4µm pore).
8. Vibratome.
9. Carbogen (95% O_2 + 5% CO_2)/-cell culture incubator.
10. Pasteur pipette.

2.3. OBSCs Fixation

1. Phosphate-buffered saline (PBS, 1X), pH to 7.5 (see **Note 1**).
2. 4% Paraformaldehyde (PFA) in PBS 1X, pH 7.5.
3. 0.1% sodium azide in PBS 1X.
4. Parafilm.

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2.4. Immunofluorescent-Immunohistochemistry (IF-IHC)

1. 24-well plate.
2. Scalpel.
3. PBS 1X.
4. Blocking/permeabilization buffer with PBS 1X, 0.1% Triton X-100 and 2% normal goat serum.
5. Antibody buffer solution: PBS 1X, 0.1% Triton X-100 and 2% normal goat serum (for primary antibodies)/2% NGS in PBS 1X (for secondary antibodies and tertiary staining solution) (see **Note 2**).
6. Primary antibodies:
   a. Mouse monoclonal anti-glial fibrillary acidic protein (GFAP) antibody (1:400, e.g., Sigma–Aldrich Chemicals G3893) to label the astrocytes.
   b. Rabbit anti- Lysosomal-associated membrane protein 1 (LAMP1) antibody (1:100, e.g., Abcam ab24170) to label lysosomes within the cells.
   c. Chicken anti-synaptophysin antibody (1:500, e.g., SySy 101006) to label presynaptic puncta.
7. Secondary antibodies:
   a. Cy3-conjugated anti-mouse IgG antibody (1:400, e.g., Thermo Scientific A10521)
   b. Biotin-conjugated anti-rabbit IgG antibody (1:500 e.g., Jackson lab 11-065-003)
   c. Alexa Fluor™ 488-conjugated goat anti-chicken IgG antibody, (1:500 e.g., Invitrogen A11035)
8. Tertiary staining molecules:

a. Alexa Fluor™ 647-conjugated Streptavidin (1:1000 e.g., Invitrogen S21374)
b. 4′,6-diamidino-2-phenylindole (DAPI, 1:1000 e.g., Sigma D9542) to label nuclei

9. Mounting medium (e.g., DAKO S3023)
10. Narrow pattern forceps (straight).
11. Microscope slides.
12. Microscope cover glass.
13. Pipettes and other general laboratory equipment.
14. Horizontal shaker.
15. Confocal microscope.

2.5. Purification of the Proteins from OBSCs

1. RIPA buffer with Tris-HCl pH 7,5 (50mM), NaCl (150 mM), SDS (0,1%), Triton X-100 (1%), Sodium Deoxycholate (0,5%), Protease Inhibitors (10 ml), Millipore H_20.
2. PBS 1X.
3. 1,5 ml Eppendorf tubes.
4. Narrow curved forceps.
5. Sonicator/Homogenizer.
6. Centrifuge.

2.6. Immunoblotting

1. SDS-PAGE gels:
   a. Millipore H_20.
   b. Acrylamide
   c. APS
   d. TEMED
2. 5X Laemmli Buffer with Trizma base (TRIS ,0,3 M), Sodium dodecyl sulphate (SDS, 10%), Glycerin (5 M), Ethylenediaminetetraacetic acid (EDTA, 25 mM), DL-Dithiothreitol (DTT, 0,5 M), Bromphenol Blue (0,5 mM).
3. 5X Running buffer with Glycine (959 mM), TRIS (123 mM), SDS (17 mM), Millipore H20. Adjust pH to 8.3.
4. Transfer Buffer with Glycine (150 mM), TRIS (20 mM), Methanol (20%), Millipore H20. Adjust pH to 8.3.
5. TBST with TRIS (10 mM), NaCL (150 mM), TWEEN 20 (0,1%), Millipore H20. Adjust pH to 7.4.
6. Protein marker (pre-stained).

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7. Western Blotting Equipment: chambers, power source, filter papers, foam pads, cassettes, polyvinylidene fluoride (PVDF) membranes.
8. Methanol.
9. 5% Milk/BSA (Bovine serum albumin) in TBST.
10. 1,5 ml Eppendorf tubes.
11. Primary antibodies:
    a. Rabbit anti-multiple EGF-like-domains 10 (MEGF10) antibody (1:500, e.g., Thermofisher).
    b. Mouse anti-postsynaptic density protein 95 (PSD95) antibody (1:2500, e.g., Sigma-Aldrich) as a marker of phagocytic astrocytes.
    c. Rabbit anti-synaptophysin antibody (1:5000, e.g., Abcam).
    d. Rabbit anti-β-actin antibody (1:10,000, e.g., Abcam) as a loading control.
    e. Rabbit anti-cofilin monoclonal antibody (1:1000, e.g., Cell Signaling) as a loading control.
12. Secondary antibodies:
    a. Horseradish peroxidase (HRP)-conjugated anti-mouse (1: 2500, e.g., Dianova).
    b. Biotinylated anti-rabbit IgG antibody (1:500/1:2500/1:5000/1:10000, e.g., Jackson Lab).
13. Enhanced chemiluminescence (ECL) substrate.
14. ImageJ software.

3. Methods

3.1. Preparation of Organotypic Brain Slice Cultures (OBSCs)

1. Pre-incubate inserts in culture medium at least 2 hours in advance (or late the day before). Add 1mL of culture medium into each well and transfer inserts using forceps into the 6-well-plate (see **Note 3)**.
2. For the preparation of brain slices, decapitate rat pups of 4-7 PND with a rough cut using sharp scissors, keeping the head submerged in carbogenated ice-cold dissection medium (see **Note 4)**.
3. Expose and open the skull, placing the brain into disection medium, carefully remove meninges and blood vessels of the brain using narrow pattern forceps. Using scalpel, remove the olfactory bulb and cerebellum with a rough-cut, creating a flat surface for embedding the brain to cutting disk. Mount the brains with a thin layer of superglue.
4. Cut coronal slices containing the PFC (400 μm thickness, 9 slices of both hemispheres) using a vibratome platform submerged in cold carbogenated dissection medium at a speed of 0.06 mm/s. Transfer 3 slices per well into 6-well-plate filled with dissection medium (see **Note 5).**
5. Under sterile conditions, place the slices onto the inserts in preincubated with the culture medium 6-well-plate, ensuring slices are not submerged but exposed to air at the surface, (maximum 3 slices per insert) (see **Note 6).** Incubate at 37°C in 5% CO_2-cell culture incubator.
6. To keep OBSCs in culture up to 21 days in vitro (DIV), replace half of the OBSCs culture medium (500 μL removed, replaced with 800 μL due to evaporation) every 2-3 days.

3.2. Preparation of OBSCs for Immunofluorescent-Immunohistochemistry (IF-IHC)

1. Transfer membranes containing brain slices into a 6-well plate and wash with 1-2 ml PBS 1X for 10 min. Separate each slice by cutting the membrane using scalpel and place in wells filled with 1.2 ml 4% paraformaldehyde in PBS 1X, leave gently shaking overnight (ON) at 4°C.
2. Place the slices into new 6-well-plate washing them three times with PBS 1X at room temperature (RT). Finally, transfer membranes with the slices into wells with 1.5 ml 0.1% sodium azide in PBS solution. Seal the plate with parafilm and store at 4°C.
3. Procedure for the IF-IHC
4. Remove the plates from the fridge, place slices from each brain in one well of a 12-well plate with about 2 ml of PBS1X per well and let them swim on a horizontal shaker for 10 minutes (repeat 3 times).
5. Incubate slices in "Blocking/permeabilization buffer" for 2 hours at RT.
6. Remove the "Blocking/permeabilization buffer" and place slices in 200 μL "Antibody buffer solution" with primary antibodies ON horizontally shaking at 4°C (see **Note 7**).
7. Wash slices with PBS 1X 3 times for 20 minutes each.

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8. Remove PBS solution and incubate slices in 200 µL "Antibody buffer solution" with secondary antibodies for 2 hrs on a horizontal shaker at RT (see **Note 8**).
9. Remove the antibody solution and wash slices with PBS 1X 3 times for 10 minutes each.
10. Remove the "Washing buffer" and incubate slices in "Antibody buffer solution" with tertiary antibodies for 2 hrs on a horizontal shaker at RT.
11. Remove the antibody solution and wash slices with PBS 1X 3 times for 10 minutes each.
12. Using forceps (grab the membrane, avoid destruction of the slice) place slices from one brain on the top of the microscope slides facing the same direction and let slices to air-dry for a few minutes under an aluminium foil.
13. Put few drops of mounting medium on slides and cover them with a microscope cover glass (see **Note 9**).
14. Keep sections in a refrigerator until microscopic analysis.

3.3. Confocal Microscopy

1. Select the region of interest for image capture: prelimbic and infralimbic cortex of the PFC.
2. Using a 63× oil objective, capture a minimum of six images per OBSC (30 optical sections, 0.5 µm Z-step size for each image, GFAP as a reference.) using the Zeiss LSM 880 Airyscan confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany).
3. Save files in the Imaris File Format (.ims).
4. Perform analysis on whole image area for every picture with IMARIS 9.8 software (Bitplane, Zurich, Switzerland). For that, create a co-localization channel of LAMP1+ and synaptophysin+ , mask with the GFAP+ channel. Astrocyte phagocytosis is determined as the ratio of "synphys+/LAMP1+/GFAP+" to "synphys+/LAMP1+" colocalized voxels and is further used for statistical analysis.

3.4. Protein Isolation from OBSCs

1. Remove the medium and wash in and out the inserts with ice-cold PBS1X.
2. Use forceps to scrape OBSCs from the membrane, collecting prefrontal cortex (PFC) tissue (see **Note 10**). Transfer them into tubes with 500 µL of ice-cold RIPA buffer and incubate on ice for 15 min.
3. Sonicate tissue homogenates (2 × 20 s, 20% intensity) and centrifuge at 15,000 rcf for 25 min at 4 °C. Collect the supernatant containing proteins and store at −20 °C.

3.5. Immunoblotting

1. Dilute samples in Millipore H_20 to an equal amount of total protein and denaturate with 5X Laemmli buffer at 95°C for 5 min.
2. Load samples with the total amount of 12 µg of protein and the marker into the gel (either 10% for MEGF10 or 12% separation gel for PSD95 and synaptophysin analysis – see **Note 11**). Separate them by the molecular size via electrophoresis at a constant 80V for 15 minutes and 100V for approximately 2 hours in 1X running buffer. Transfer the resolved proteins to nitrocellulose membranes for 20 minutes at 70V and then 2 hours at 110V.
3. Block the membranes either in 5% milk (β -actin, cofilin) or BSA (MEGF10, PSD95, synaptophysin) in TBST for 1 hour at RT and incubate with primary antibodies (see **Note 12**) ON at 4 °C.
4. Wash x3 membranes with TBST and incubate with respective secondary antibodies for 2 hours at RT (see **Note 12**). Wash x3 membranes with TBST again.
5. Detect and visualize protein samples by enhanced chemiluminescence, adjusting the brightness, focus, exposure, and interval time. Analyse the intensity of the bands quantitatively using ImageJ software. The immunoreactivity of the proteins of interest should be normalized to β-actin or cofilin for relative comparisons of results.

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3.6. Statistical Analysis

1. Use ANOVA test with repeated measures, as slices derived from the same brain will be analysed at different time points. Follow the analysis by Tukey's post hoc test and present data as the mean ± standard deviation (SD).
2. Statistical significance is to be considered when the p value is equal or less than 5% ($p < 0.05$).

4. Notes

1. All solutions are prepared in deionized water with a resistivity of 18.2 MΩ-cm.
2. Ideally, the serum should be derived from the same species in which the secondary antibody is raised. In case of IF-IHC with multiple secondary antibodies derived from different species, we have got good experiences with the normal goat serum.
3. While transferring culture inserts into the wells filled with OBSCs culture medium, avoid trapped bubbles beneath (by moving the insert with forceps).
4. Dissection medium must be ice-cold during dissection and sectioning to prevent nucleic acid degradation and maintain the tissue as much as possible [20].
5. The explants consist of 400µm/coronal slices of the rostral end of the rodent brains, giving a high resolution. We suggest cutting nine slices per each hemisphere, distributing them into three wells in a following manner that would be ideal for a complete representation of the different regions of the brain:
   a. Well A1: slice 1,4,7
   b. Well A2: slice 2,5,8
   c. Well A3: slice 3,6,9

   Keep slices in incubator cutting the next brain meanwhile, submerged in the medium no longer than 3 hours.
6. While replacing the slices onto inserts make sure no bubbles are trapped under and no medium covers the slices.
7. During the change of solutions, slices should never get dried.
8. As secondary/tertiary antibodies are light-sensitive, plates must be covered with an aluminium foil to keep the fluorescent dyes protected from light.
9. Be careful not to form bubbles in the mounting medium as they might interfere with imaging at the confocal microscope. In case bubbles form, you can gently pull them out with the help of a pipette tip pressed on the cover slip.
10. It is doubtful to selectively isolate only PFC from other cortical regions, therefore the tissue may contain proteins from neighbouring areas.
11. The choice of the concentration of the gel is based on target's molecular weight in which protein would resolve better.
12. Antibodies are diluted in 2,5% BSA-TBST (MEGF10), 5% BSA-TBST (PSD95, synaptophysin), 5% milk-TBST (β -actin, cofilin). The concentration of antibody collusion buffer depends on the specific antibody and can be adjusted.

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